Cytochrome P-450 Regulation in Human Tumour-derived Cell Lines.

This thesis is my own composition and describes a project carried out by myself; experiments performed by other people are appropriately acknowledged.

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PhD.
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1990.

For the Lord gives wisdom,
and from his mouth come knowledge and
understanding.

Proverbs 2:6.

Acknowledgements.

First, let me thank Dr. Roland Wolf for supervising this project and, in the process, passing on to me some of his boundless enthusiasm for cytochrome P-450s. I would also like to thank the staff of the ICRF Laboratory of Molecular Pharmacology and Drug Metabolism for their cameraderie and helpful advice, and Sandy Bruce for his photographic expertise.

I am grateful to Prof. Andrew Miller, Dr. Nick Hastie and the staff at the Edinburgh University Department of Biochemistry and the MRC Clinical and Population Cytogenetics Unit for allowing me to participate in their stimulating seminars and journal clubs, and to the Imperial Cancer Research Fund for financing this research.

Three people, Drs. David Adams, Jim Carmichael and John Hayes, gave me invaluable help with this project: I have had many fascinating scientific discussions with them, but it is for their unique and highly individual senses of humour that I will remember them best.

I cannot complete these acknowledgements without thanking my friends in Edinburgh, particularly Una and Innes Duncan, John and Ali Schulga and Catherine Dolan, for their friendship, their patience in listening to hours of grumbling, and for providing a seemingly endless supply of coffee and chocolate!

Finally, this thesis is dedicated to my parents, without whose love and support it would most certainly never have been written.

Abstract

Cytochrome P-450s (P-450s) comprise a polymorphic multigene family of haem-containing enzymes which are essential to the Phase I metabolism of xenobiotics. They take part in activation and detoxification of carcinogens and anticancer drugs; thus an understanding of these enzymes is essential to the prevention and treatment of cancer. Induction of P-450s by drugs and carcinogens has been extensively studied; endogenous regulation of P-450s also occurs during normal development and disease. The aim of this project was to develop an in vitro system in which to study P-450 induction and modulation during inflammation. P-450 regulation in five human tumour-derived cell lines, HepG2 (liver) NCI H322, NCI H358 (lung), HT29 and LS174T (colon) was examined. The liver cell line was chosen because of this organ's importance in xenobiotic metabolism and the known effects of inflammation on hepatic P-450 expression; the lung and colon lines were selected because these organs also express P-450s and are subject to inflammatory disorders which may be involved in tumorigenesis. The cell lines expressed variable levels of 7-ethoxyresorufin O-deethylase (EROD) which could be induced by benzanthracene (BA); after BA treatment isozyme MC_{1b} could be detected by Western blot analysis. The lung cell line NCI H322 was selected for further study. Induction of isozyme MC_{1b} in this cell line involved an increase in steady-state mRNA expression; further experiments established suitable conditions for induction (5μg/ml BA in RPMI medium containing 10% foetal calf serum). The MTT assay was used to estimate the toxicity of P-450 inducers towards HepG2 and NCI H322 cells and to examine the effects of P-450 induction on the toxicity of benzo(a)pyrene (B(a)P) and cyclophosphamide. The results of cytotoxicity assays using B(a)P were unexpected in that, rather than increasing the cells' susceptibility to B(a)P toxicity, pretreatment with BA was protective for HepG2 cells; a mechanism involving induction of detoxifying enzymes was proposed. Assays using cyclophosphamide confirmed that both cell lines were capable of activating this drug; induction of MC_{1b} did not affect susceptibility to cyclophosphamide. The response of NCI H322 cells to inflammatory mediators was compared with that of the CBA mouse liver. In the mouse, constitutive P-450 expression was suppressed by a high dose of endotoxin (25μg/day) and by interferon α, but P-450 induction by 3-methylcholanthrene and phenobarbital appeared to be potentiated by low doses of endotoxin. The effects of endotoxin, dexamethasone and five recombinant DNA-derived cytokines, interleukin-1β, tumour necrosis factor, and interferons α, β and γ, on constitutive and BA-induced EROD were examined in NCI H322 cells. No evidence for potentiation of P-450 induction was observed; the most dramatic effect observed was that of ifn γ which suppressed EROD to 47.5% and 27.7% of the non-ifn γ-treated sample in controls and BA-treated cells, respectively; this suppression was not due to toxicity. It is hoped that the experiments described in this thesis will establish the usefulness of human tumour-derived cell lines of extrahepatic origin in studying P-450 regulation. The use of continuously cultured cell lines complements in vivo studies by making it possible to examine the direct effects of specific mediators in a closely defined system. Such an approach has the advantage of simplicity compared with the in vivo approach, although all results must be confirmed in vivo; an integrated approach using both continuously cultured cell lines and experimental animals can be both informative and economical.

Contents.

Abbreviations.

AHH:	Aryl hydrocarbon hydroxylase.
ALA:	5-aminolevulinic acid.
AMP:	Adenosine 5'-monophosphate (cAMP: cyclic AMP)
APS:	Ammonium persulphate.
ATP:	Adenosine 5'-triphosphate (dATP: deoxyadenosine 5'-triphosphate)
BA:	Benzanthracene. (DMBA: dimethylbenzanthracene)
B(a)P:	Benzo(a)pyrene. (3-OHB(a)P: 3-hydroxybenzo(a)pyrene).
BCG:	Bacillus Calmette Guerin.
7-BR:	7-benzyloxyresorufin. (BROD: 7-benzyloxyresorufin O-deethylase).
CDTA:	Trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid.
CTP:	Cytidine 5'-triphosphate (dCTP: deoxycytidine 5'-triphosphate)
Dex:	Dexamethasone.
DMEM:	Dulbecco's modification of Eagle's medium.
DMSO:	Dimethylsulphoxide.
DNA:	Deoxyribonucleic acid. (cDNA: Complementary DNA.)
7-EC:	7-ethoxycoumarin. (7-OHC: 7-hydroxycoumarin).
EDTA:	Ethylene diamine tetraacetic acid.
EH:	Epoxide hydrolase.
7-ER:	7-ethoxyresorufin. (EROD: 7-ethoxyresorufin O-deethylase).
FAD:	Flavin adenine dinucleotide.
FMN:	Flavin mononucleotide.
GDP:	Guanosine 5'-diphosphate.
GST:	Glutathione S-transferase.
GTP:	Guanosine 5'-triphosphate (dGTP: deoxyguanosine 5'-triphosphate)
HEPES:	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid].
Ifn:	Interferon.
IL-1:	Interleukin-1.
Kd:	Dissociation constant.
3-MC:	3-methylcholanthrene.
MEM:	Minimal essential medium.
Mr:	Relative molecular weight.
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
NADPH:	β-nicotinamide adenine dinucleotide phosphate (reduced form).
P-450:	Cytochrome P-450.
PAH:	Polycyclic aromatic hydrocarbon.
PB:	Phenobarbital.
PBS:	Phosphate buffered saline.
PCN:	Pregnenolone-16α-carbonitrile.
Poly IC:	Polyriboinosinic acid-polyribocytidylic acid.
7-PR:	7-pentoxyresorufin. (PROD: 7-pentoxyresorufin O-deethylase).
RNA:	Ribonucleic acid (mRNA: messenger ribonucleic acid).
RPMI:	Roswell Park Memorial Institute.
SD:	Standard deviation from the mean.
SDS-PAGE:	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis.
SEM:	Standard error of the mean.
SSC:	150mM sodium chloride, 15mM sodium citrate.
TAE:	Tris-acetate-EDTA.
TBS:	Tris-buffered saline (TBST: TBS + 0.05% Tween 20)
TCDD:	2,3,7,8-tetrachlorodibenzo(p)dioxin.
TEMED:	N,N,N',N'-tetramethylethylenediamine.
TNF:	Tumour necrosis factor.
TTP:	Thymidine 5'-triphosphate (dTTP: deoxythymidine 5'-triphosphate)
UDPGT:	uridine diphosphate-glucuronyl transferase.
WEM:	William's E medium.

Chapter 1.
General Introduction.

1.1. Introductory remarks.

Among the most important routes by which cells metabolise xenobiotics is that involving the cytochrome P-450-dependent monooxygenase system. Cytochrome P-450s (P-450s) comprise a polymorphic multigene family of haem-containing enzymes which are essential to the Phase I metabolism of drugs and carcinogens (Wolf (1986)). The P-450s may participate in either activation or detoxification of carcinogens and anticancer drugs (Guengerich (1988)); thus an understanding of these enzymes is essential to any programme aimed at the prevention or treatment of cancer. P-450s are subject to regulation by a wide range of exogenous and endogenous factors. Induction of P-450s by drugs and carcinogens has been extensively studied ever since the identification of these cytochromes some 30 years ago; endogenous regulation of P-450s also occurs during normal development and in various disease states. The aim of this project was to develop an in vitro system in which to study P-450 induction together with one aspect of the endogenous regulation of P-450s, their modulation during infection and inflammation.

In this chapter the literature pertaining to regulation of P-450-dependent drug metabolism during inflammation will be introduced. In order to do this it will be necessary to describe the properties of the P-450-dependent monooxygenase system and its regulation by xenobiotics, but the extensive literature in this area will not be discussed in detail: the reader is referred to a number of recent reviews which cover this area. The majority of the chapter will be devoted to a review of the evidence concerning the regulation of P-450-dependent drug metabolism during infection and inflammation. A number of questions concerning this aspect of drug metabolism will be raised, and the strategy to be adopted in attempting to answer them will be described. In subsequent chapters the background to different aspects of the project will be discussed in detail, and the final chapter will summarise the findings of the project and suggest some areas for future research.

1.2. Introduction to cytochrome P-450s.

1.2.1. P-450 enzymology.

In the late 1940s, James and Elizabeth Miller and their associates identified a group of NADPH-dependent microsomal enzymes involved in the biotransformation of azo-dyes (Conney et al (1956), Conney 1986)). The reactions catalysed by these enzymes also proved to be important in the processing of other carcinogens as well as drugs and steroids, and subsequent work identified the agent responsible for this activity as a liver pigment which bound carbon monoxide. This pigment, a b-type cytochrome containing iron protoporphyrin IX as a prosthetic group, was named "cytochrome P-450" because the complex between the reduced pigment and carbon monoxide had an absorption maximum of 450nm (Omura and Sato (1964)).

The P-450-dependent metabolism of xenobiotics involves two phases, Phase I and Phase II. In Phase I a highly lipophilic compound such as a polycyclic aromatic hydrocarbon (PAH) is oxidised by addition of a hydroxyl or epoxide group; in Phase II the resulting highly reactive electrophile is conjugated to a hydrophilic group such as glutathione, making it sufficiently water-soluble for transport in the bloodstream and excretion in the urine (Nebert and Negishi (1984)).

Drug Carcinogen Steroid	Phase I ——————> P-450	Reactive Intermediate eg. epoxide	Phase II ——————> eg. GST	Hydrophilic Conjugate eg. glutathione conjugate

Although P-450-mediated metabolism is essential to the detoxification of many compounds, the highly reactive intermediates formed during this process are often more toxic than the parent compound and have the capacity to bind to cellular macromolecules such as proteins and DNA, causing severe cellular damage. This process often results in mutation and can lead to carcinogenesis (Conney (1986))

The mechanism of P-450-mediated catalysis is complex and is not yet fully understood (Groves (1986)). P-450 is the terminal oxidase of a microsomal electron transport chain in which two electrons are passed from NADPH (reduced nicotinamide adenine diphosphate) via NADPH-cytochrome P-450 reductase (hereafter referred to as reductase) and P-450 to mediate the incorporation of one

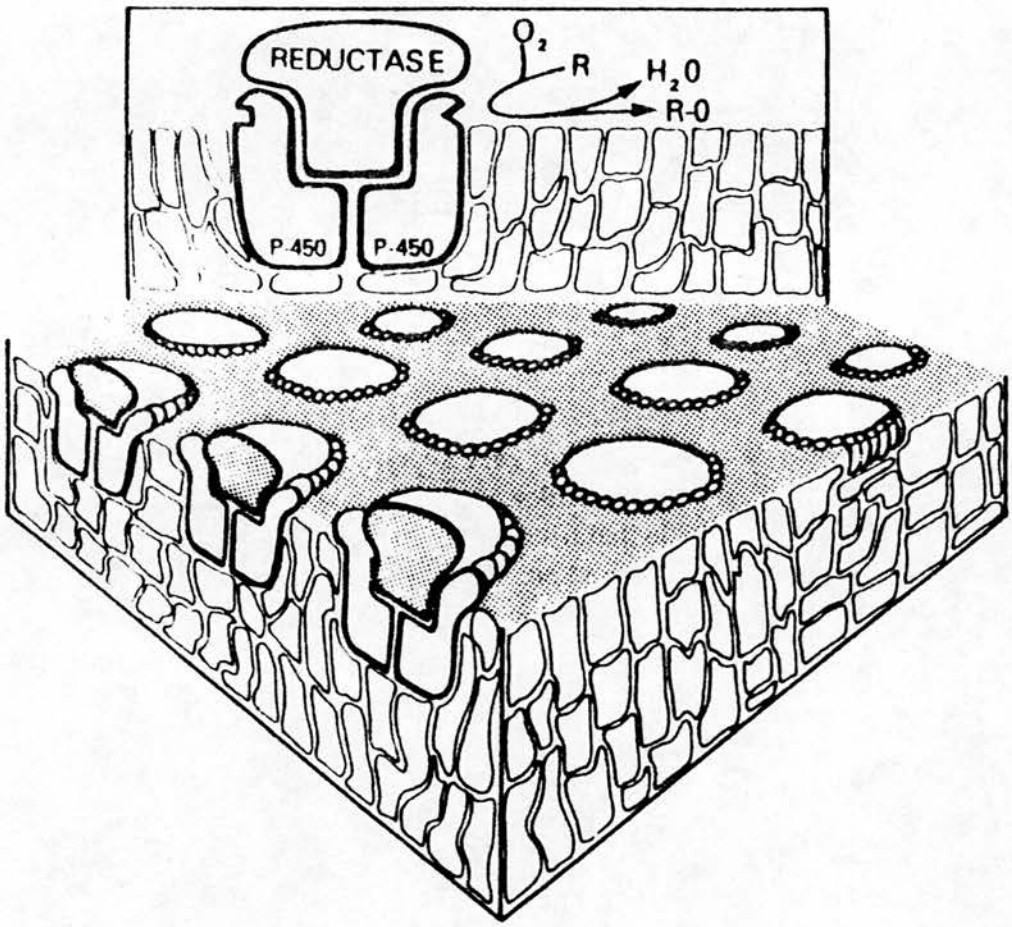
atom from molecular oxygen into a carbon-hydrogen or double carbon-carbon bond in the substrate: the monooxygenase reaction (Black and Coon (1987)). During electron transport, cytochrome P-450 interacts with reductase, a protein of molecular weight 77,700 (in the rabbit liver) (Black et al (1979)), which is associated with the endoplasmic reticular membrane and contains one flavin mononucleotide (FMN) and one flavin adenine dinucleotide (FAD) molecule per catalytic unit (Iyanagi and Mason (1973)). The stoichiometry of the interaction between P-450 and reductase is not understood: the P-450 and reductase molecules may be permanently associated with each other in a multienzyme complex, or may meet during lateral diffusion through the membrane (Nebert and Gonzalez (1987)). One model for a P-450-containing multienzyme complex is shown in Figure 1.1. The interaction between the two enzymes appears to be mediated by a small hydrophobic region of the reductase molecule which binds it to the membrane and to P-450 whilst the larger, more hydrophilic domain is exposed to the cytosol and is the catalytic domain of the enzyme (Black et al (1979)). Electrons are passed from FAD to FMN within the reductase molecule and then to P-450 leading to the incorporation of one atom from molecular oxygen into the substrate whilst the other oxygen atom is reduced to form water (Black and Coon (1987)).

"Cytochrome P-450" is not a single entity but comprises a superfamily of related enzymes which are widely distributed in animals, plants and protists (Black and Coon (1987)). Mammalian P-450s are integral membrane proteins of molecular weight 50,000 - 60,000 which are found in high concentrations in the endoplasmic reticulum (Dallner and DePierre (1982)) and in lower concentrations in the mitochondrial, nuclear and plasma membranes (Oesch et al (1985)). The organ containing the highest concentration of P-450 is the liver; however, organs which are accessible to compounds from the environment, including skin, gastrointestinal tract and lung, contain significant levels of some P-450s (Bend and Hook (1974)).

The members of the cytochrome P-450 superfamily have broad, overlapping specificities with regard to both the substrates metabolised and the sites of attack within the substrate molecule (Lu and West (1980)). Many biotransformation reactions involving both xenobiotic and endogenous substrates are dependent on P-450 catalysis. The most common reaction catalysed is monooxygenation, but P-450s can also mediate epoxidation, peroxygenation, N-, S- and O-dealkylation, N- and S-oxidation, dehalogenation, desulphurisation, reduction of nitro-, azo-,

Figure 1.1.

Three-dimensional concept of the monooxygenase system in the endoplasmic reticulum.



R = substrate.
From Nebert and Gonzalez (1987)

and N-oxide groups, peroxides and epoxides, and deamination (Black and Coon (1987)). The xenobiotic substrates metabolised include many drugs, carcinogens and model substrates; endogenous P-450 substrates include fatty acids (ω, ω - 1 hydroxylation), prostaglandins, leukotrienes and thromboxanes (isomerisation, ω-hydroxylations involved in biosynthesis and metabolism), steroid hormones and bile acids (hydroxylations) (Wolf (1986)).

Improvements in purification techniques made it possible to study the properties and regulation of specific P-450 isozymes. In order to purify the P-450s it is necessary to solubilise the microsomal membrane using non-denaturing detergents such as Emulgen 911, Lubrol, cholate or deoxycholate. The P-450s may then be separated according to hydrophobicity (on Octylamino-Sepharose or Lauryl-Sepharose) and by anion exchange (on DEAE-cellulose). Hydroxylapatite chromatography may be used as an additional separative technique (Astrom and DePierre (1986)). A number of biochemical methods have been used to characterise the multiple P-450s: one commonly used criterion for identifying P-450s is mobility on SDS-PAGE, although this is not sufficient for conclusive identification because of differences in the electrophoretic systems used by different laboratories. Detailed characterisation may be performed using polyclonal antisera raised against specific P-450 isozymes, together with spectrophotometric and enzymological techniques (Guengerich et al (1981, 1982a,b)). However, all biochemical studies on P-450s must be interpreted with caution because of the difficulty of obtaining, and demonstrating that one has obtained, a pure, homogeneous P-450 preparation, a requirement which is important in spectrophotometric and enzymological studies and essential to the production of monospecific polyclonal antisera.

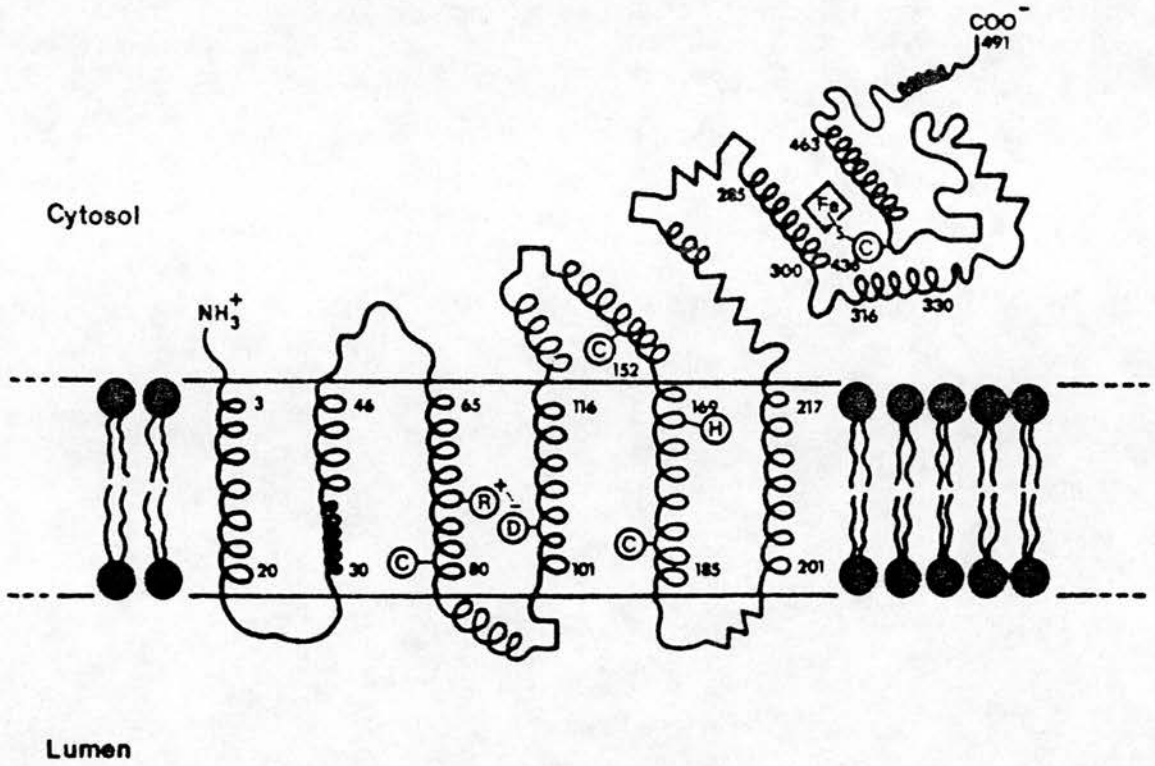
It was only with the development of new techniques in monoclonal antibody technology (Gelboin et al (1984)) and molecular biology (Nebert et al (1984)) that it became possible to study in detail the structure and regulation of different P-450s and the relationships between them. Recent developments in amino-terminal sequencing of purified proteins and complete sequencing of cDNA clones have allowed a number of properties of P-450 molecules to be characterised and their evolutionary relationships to be more fully understood (Nebert and Gonzalez (1987)). The P-450 superfamily is thought to be derived from a single ancestral gene which may have existed early in evolution: P-450s have been characterised in some very primitive

organisms including the bacterium Pseudomonas putida, whose camphor-metabolising enzyme was the first P-450 to have its 3-dimensional structure determined by x-ray crystallography (Poulos et al (1985)). The P-450 superfamily is thought to have arisen as a result of a series of duplication events; rapid evolution of this family occurred, especially after the divergence of the mammals (Wolf (1986)). The number of P-450s is so large that it was suggested that their diversity might be generated by a somatic recombination mechanism similar to that of the immunoglobulin superfamily (Nebert (1979)); however, there is no evidence in support of this hypothesis.

In spite of the rapid evolution of P-450s, all members of this superfamily retain a number of features in common. Black and Coon (1987) compared many P-450 sequences reported up to 1986. This analysis showed that the P-450 molecules studied consisted of 414 - 524 amino acid residues, with similar compositions including at least 4 cysteine residues. The molecular weights of the P-450s were narrowly distributed about a mean value of 57,000. Three regions of homology were found, centred around two cysteine residues (cys_{152} and cys_{438} of rabbit P-450 Form 2) and around residue 390 (again of Form 2). The cysteine residues are thought to be important in the structure and catalytic activity of P-450s; one of them may act as the proximate haem thiolate ligand (Gotoh et al (1983)). The sequences involved in other essential functions, such as interaction with reductase, have not yet been identified. Sequence data, together with fluorescence energy transfer experiments, suggest that P-450s such as rabbit Form 2 cross the membrane several times with polar segments protruding on one or both sides of the bilayer. Figure 1.2 shows the interaction between rabbit P-450 Form 2 and the microsomal membrane: it proposes that the haem moiety is chelated to cys_{436} in a globular cytoplasmic domain whilst the N-terminal region of the molecule binds to the membrane by six transmembrane sequences (Black and Coon (1987)). Similar results were obtained in structure prediction studies of rat P-450 Form d, which contains 17 helical regions, four of which are sufficiently hydrophobic to cross the lipid bilayer whilst the rest are amphiphilic. Analysis of the protein with respect to membrane topology indicated the presence of globular cytoplasmic domains anchored to the membrane by helices at the N- and C-termini, with a possible third anchoring region near to the centre of the sequence (Haniu et al (1986)).

Figure 1.2.

Proposed model of the membrane topology of rabbit P-450 isozyme 2.



From Black and Coon (1987).

1.2.2. Classification of P-450s.

Cytochrome P-450s were initially classified into families according to induction by various xenobiotics. On this basis, families inducible by the "classical" inducing agents phenobarbital (PB) and 3-methylcholanthrene (3-MC) were identified.

These compounds are by no means the only inducers of P-450s; other "PB-like" and "3-MC-like" inducing agents exist, metyrapone being a PB-like inducer, whilst 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (B(a)P), and benzanthracene (BA) are 3-MC-like inducers. A variety of other classes of inducing agents including glucocorticoids (eg. dexamathasone (dex), pregnenolone-16a-carbonitrile (PCN)), alcohols (eg. ethanol) and peroxisome proliferators (eg. clofibric acid) have also been identified (Nebert and Gonzalez (1987)). Cloning of a number of P-450s made it clear that P-450 families are related not only by inducibility but also at the level of sequence homology, and P-450s are now classified into gene families on this basis. Alignment of amino acid sequences from a number of P-450s indicated that at least 8 mammalian P-450 gene families exist, separate families being genetically unlinked and often found on different chromosomes. There is less than 36% sequence homology between members of different gene families, whereas members of the same family share ≥40% homology and members of the same subfamily are defined as being over 70% homologous (Nebert et al (1987)).

Generally such a classification yields the same results as classification according to inducibility, though in some cases the older system is inadequate: for example, many members of the "PB-inducible" gene family are only marginally inducible (Nebert and Gonzalez (1987), Wolf et al (1984,1986)). A variety of nomenclatures have been used to classify P-450 gene families; indeed, a great deal of confusion has arisen as a result of the use of different nomenclatures by almost every laboratory in the field. In the "recommended nomenclature" recently proposed by Nebert et al (1987) every gene family is given a number:

eg.	Family I	"3-MC-inducible"
	Family II	"PB-inducible"
	Family III	"Steroid-inducible"
	Family IV	"Peroxisome proliferator-inducible"

Within a gene family, proteins are classified using letters and numbers (eg. P450IA1 is the major 3-MC-inducible P-450). A major drawback of this system is that the nomenclature of a specific P-450 is liable to change as other members of the same gene family are discovered (Nebert et al (1989)) and for this reason the

nomenclature used throughout this thesis will be that used in Dr. Wolf's laboratory, in which P-450s are identified by the compound which induces them, together with a subscript to identify the protein within its gene family: for example, MC_{1b} represents the major 3-MC-inducible P-450 in this system, and is equivalent to P450IA1. This nomenclature is related to other systems in Table 1.1.

Table 1.1.

A comparison of commonly used cytochrome P-450 nomenclatures.

P-450 Family Nebert et al	Rat Wolf	Levin	Guengerich	Other	Mouse	Rabbit
Family I						
P450IA1	MC_{1b}	c	BNF-B	P-448	P_1	Form 6
P450IA2	MC_{1a}	d	ISF-G,	-	P_3	Form 4
Family II						
Subfamily IIA						
P450IIA1	UT_1	a	UT-F	-	-	-
Subfamily IIB						
P450IIB1	PB_{3a}	b	PB-B	P-450	-	Form 2
P450IIB2	PB_{3b}	e	PB-D	-	-	-
Subfamily IIC						
P450IIC1(etc)	PB_1	k	PB-C	-	-	Form 1,
	-	-	-	-	-	Form 3b
	-	-	-	-	-	Form 5
Subfamily IID						
P450IID1	-	-	UT-H	db1	-	-
P450IID2	-	-	-	db2	-	-
Subfamily IIE						
P450IIE1	-	j	-	-	-	Form 3a
Family III						
P450IIIA1	PB_{2c}	-	PCN-E	pcn1	-	Form 3c
P450IIIA2	-	-	-	pcn2	-	-
Family IV						
P450IVA1	Clo	-	-	LA_{ω}, P-452		-

In this table the system described in the 1987 paper of Nebert et al has been used; a recent update (Nebert et al (1989)) made changes to the system, in that the root symbols CYP (for human P-450) and cyp (for mouse P-450) were introduced, and Roman numerals were replaced by Arabic numbers. Since this system is still subject to frequent alteration the 1987 system will be used whereever the nomenclature of Nebert et al is referred to in this project.

1.3. Regulation of P-450 expression by xenobiotics.

1.3.1. The P450I gene family: PAH-inducible P-450s.

Regulation of polycylic aromatic hydrocarbon (PAH)-responsive P-450 genes has been the subject of intense research ever since the observation that PAH treatment stimulates the metabolism of carcinogens by increasing the rate of synthesis of the enzyme responsible, aryl hydrocarbon hydroxylase (AHH) (Conney et al (1956)). This research has revealed that in the rat, rabbit and mouse the PAH-inducible gene family (Family I) comprises two members; in the rat these are called MC_{1a} and MC_{1b}. Both isozymes are induced by PAHs such as 3-MC, B(a)P and BA, polychlorinated biphenyls such as Aroclor 1254 and flavones such as β-naphthoflavone; however, the constitutive level of MC_{1a} is much higher than that of MC_{1b}, and this isozyme is preferentially induced by isosafrole, indicating that the two enzymes are not coordinately regulated (Ryan et al (1979, 1980), Reik et al (1982), Kawajiri et al (1984)). The major 3-MC inducible P-450, MC_{1b}, is highly active in metabolism of PAHs such as B(a)P, BA and dimethyl benzanthracene (DMBA) (Conney et al (1957), Wood et al (1976a), Conney (1982)) as well as model substrates such as 7-ethoxy-resorufin (Burke and Mayer (1974)). Many of these PAHs are potent carcinogens, and P-450-mediated metabolism may result in activation or detoxification (Gelboin (1980), Conney (1982)). The regulation of this class of P-450s is therefore very important in determining susceptibility to chemical carcinogenesis.

In both the rat and the mouse, MC_{1a} and MC_{1b} are induced at the mRNA level by treatment with PAHs (Whitlock (1986)). The regulatory system involved has been studied in detail in the mouse: this was possible because of the availability of inbred strains of mice having differing sensitivity to PAHs, expressed in a number of ways including differences in susceptibility to DMBA-induced skin inflammation and B(a)P-induced skin tumorigenesis as well as differences in hepatic 3-MC-inducible AHH activity (Eisen et al (1983)). Some mouse strains (eg C57BL/6N) are highly responsive to PAHs whilst others, (eg. DBA/2N) are non-responsive. This difference is regulated by a locus called the Ah locus (for Aryl hydrocarbon responsiveness); PAH-responsiveness segregates as an autosomal dominant trait between these strains, and this finding led to the proposal that responsiveness depends on a factor such as a receptor molecule encoded by the Ah locus. However, later studies on a larger number

of mouse strains showed that inheritance of Ah responsiveness is complex, involving several genes which show co-dominant effects (Nebert and Negishi (1984)). Use of the highly potent AHH inducer TCDD showed that apparently non-responsive mouse strains could respond to induction but only at concentrations of TCDD 10 -20 times those to which C57BL/6N mice responded. It was found that the receptor in hepatic cytosol of C57BL/6N mice had a dissociation constant for TCDD of 2.7×10^{-10}M, whereas the dissociation constant of the DBA/2N receptor was at least 50 times higher. The dissociation constant of the DBA/2N receptor was difficult to quantify because the concentrations required to do so exceeded the solubility of TCDD in aqueous media. These data led to the conclusion that the Ah receptor was present, but defective, in DBA/2N mice, which appeared to carry a normal P_1-450 gene (Poland et al (1976)).

The Ah receptor appears to mediate the effects of PAHs by a mechanism similar to that by which steroid hormone receptors regulate steroid-responsive gene activity (Eisen et al (1983)). Briefly, the highly lipophilic PAH molecule is thought to diffuse through the plasmalemma and bind tightly to the Ah receptor. A change in the receptor's physical properties, possibly involving translocation to the nucleus, then occurs and after this receptor-ligand complexes may be detected in the nucleus. Nuclear eventsn which are not fully understood, but may involve interaction with specific DNA sequences at the 5' end of the P-450 gene, then occur causing an increase in the rate of transcription of specific genes such as P_1-450. The mRNA produced is translated and the resulting apoprotein binds haem forming the active AHH enzyme.

The properties of the Ah receptor have been characterised using TCDD as a model ligand. TCDD is ideal for studies of this kind because it is the most potent known inducer of Ah associated structural gene products, is only minimally metabolised by AHH and can be radiolabelled to high specific activity (Eisen et al (1983)). Use of this ligand revéaled that TCDD binding to membranes is non-specific and non-saturable, whereas hepatic cytosol contains a small number of high affinity, saturable TCDD binding sites. Using AHH induction as a marker it was shown that the potency of inducing agents correlated closely with their ability to displace labelled TCDD from these cytosolic binding sites (Okey et al (1979)).

The exact molecular events following binding of TCDD to the Ah receptor are not yet fully understood. This problem is currently under investigation in cell culture models,

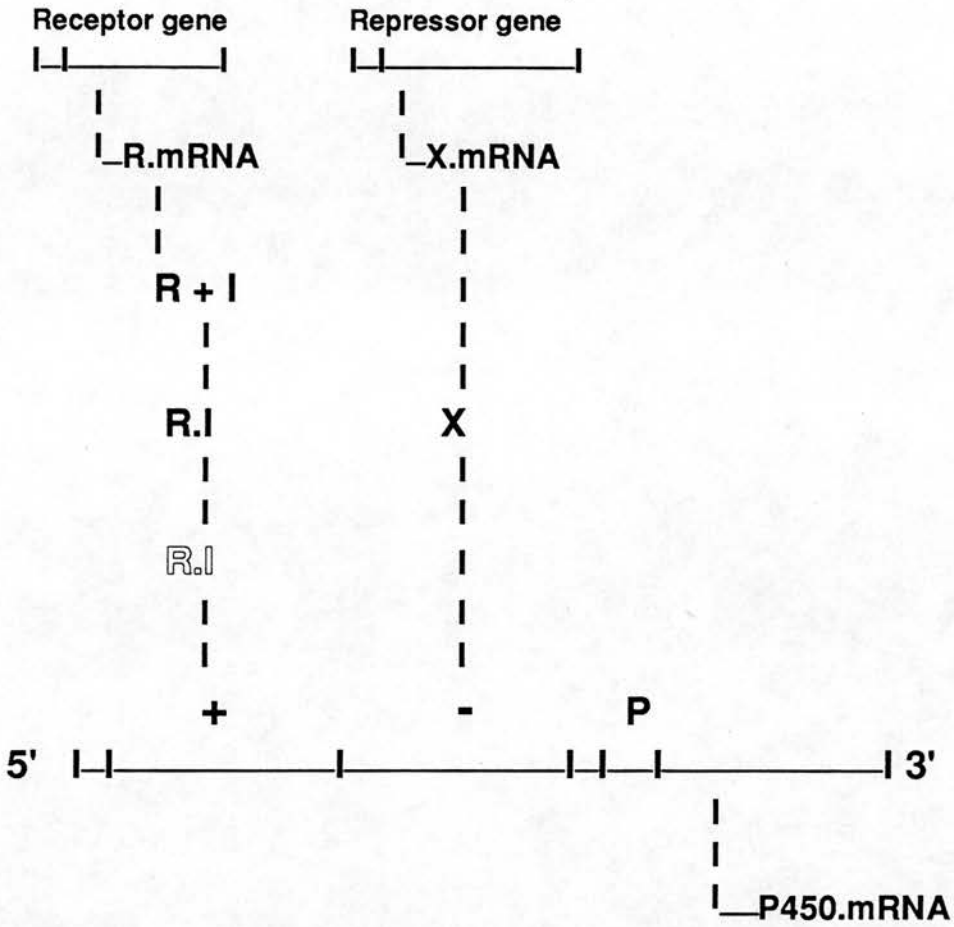
particularly the mouse hepatoma cell line Hepa-1. This cell line has been shown to contain a functional Ah receptor which binds TCDD and other PAHs with high affinity ($Kd = 4.5 \times 10^{-10}M$) and has similar properties to the sodium-molybdate-stabilised glucocorticoid receptor, as does the Ah receptor of rat liver (Cuthill et al (1986), Wilhelmsson et al (1986)). Circumstantial evidence, such as the appearance of detectable nuclear receptor sites only after TCDD treatment, indicates that following binding of inducing agent to the receptor the inducer-receptor complex may undergo translocation from the cytosol to the nucleus (Okey et al (1979, 1980), Tukey et al (1982)). However, as with the steroid hormone receptors, nuclear translocation of the receptor has not been conclusively demonstrated and it is possible that following TCDD treatment, latent receptors in the nucleus become available for binding (Whitlock and Galeazzi(1984)).

On reaching, or being formed within, the Hepa-1 cell nucleus, the inducer -receptor complex binds to a DNA sequence upstream from the P_1-450 gene (Jones et al (1984)). In transfection experiments this sequence conferred PAH-inducibility on the chloramphenicol acetyl transferase gene (Jones et al (1985)). Expression of hybrid human and rat $P_1$450-chloramphenicol acetyl transferase genes in Hepa-1 cells showed that these genes also contain 5' drug responsive elements (Kawajiri et al (1986), Sogawa et al (1986)). At least one other element, a repressor of P-450 synthesis, may also be involved: studies on this aspect of P-450 regulation in Hepa-1 cells indicated that both positive and negative regulation of MC_{1b} expression can occur (Hankinson et al (1985)). Whitlock's group demonstrated the existence of a putative repressor binding site as well as the TCDD-responsive sequence in the 5' region of the gene (Jones et al (1984, 1985)). They proposed that Ah receptor binding enhances expression by releasing the MC_{1b} gene from endogenous repression. Whitlock's model for MC_{1b} (P_1-450) regulation in mouse hepatoma cell lines is shown in Figure 1.3.

A number of suggestions have been made concerning the physiological role of the Ah receptor system. One possibility is that the system arose to deal with combustion products and plant toxins; however, since many naturally occuring compounds, such as aflatoxin B_1, are activated to potent carcinogens by this P-450 family it is hard to see what overall benefit such a system might confer (Eisen et al (1983)). Although all the Ah receptor ligands identified to date have been xenobiotics, it is also possible that

Figure 1.3.

A model for the regulation of cytochrome P_1-450 expression in mouse hepatoma cells.



The inducer (I) forms a complex (R.I) with an intracellular receptor (R) which is encoded by a gene at the Ah locus. The inducer-receptor complex undergoes a temperature-dependent change to a form (R.I) which binds tightly to chromatin. The binding of the inducer-receptor complex to a genomic domain (+) upstream of the promoter (P) stimulates cytochrome P_1-450 transcription. A hypothetical, cycloheximide-sensitive labile repressor (X) binds to a second upstream genomic domain (-) and inhibits transcription. Therefore, cytochrome P_1-450 gene transcription represents a balance between positive and negative control. It is possible that additional regulatory factors and genomic control elements also exist for the cytochrome P_1-450 gene (adapted from Whitlock (1986)).

endogenous regulators bind to this receptor. If this is the case, it may have a role in normal development, since Ah ligands also induce expression of important enzymes such as alcohol dehydrogenase, phospholipase A_2, which is involved in prostaglandin synthesis, and choline and ethanolamine kinases which participate in phosphatidyl choline biosynthesis (Deitrich et al (1978), Ishidate et al (1980), Bresnik et al (1981)). Many Ah receptor ligands also cause immunosuppression (Spreafico and Vecchi (1985)). The aim of the present work was to determine whether the reverse is true: to what extent does the immune system affect the regulation of P-450 expression by the Ah receptor? There is ample evidence that constitutive P-450-dependent activities are modulated during infection and inflammation, but relatively few studies have considered the effects of infection and inflammation on the induction of P-450s. Such effects could be of great importance in determining susceptibility to drugs and carcinogens in some circumstances, such as those prevailing in the smoker's lung which is chronically exposed both to PAHs and inflammation.

1.3.2. The P450II gene family: PB-inducible P-450s.

The "PB-inducible" P-450 family (Family II) comprises several subfamilies which have been studied in detail in the rat and the rabbit. One, Subfamily IIB, contains genes which are normally expressed at low levels but are highly inducible by PB, whereas the other families are expressed at higher constitutive levels and are only marginally inducible (Wolf et al (1984,1986), Nebert and Gonzalez (1987)). Early studies on induction by PB in the rat and rabbit indicated that proteins of molecular weight 48,500 - 53,000 were induced in rat and rabbit liver following PB treatment (Lu and West (1980)). The enzyme induced had very low activity in PAH metabolism but was highly active in the N-demethylation of benzphetamine (Hewick and Fouts (1970)), depentylation of 7-pentoxyresorufin (Lubet et al (1985)) and epoxidation of aldrin (Wolff (1980)).

Clones encoding the major PB-inducible isozymes of a number of species, including rat, rabbit, chicken and mouse have been isolated and used to examine their induction (Adesnik and Atchison (1985)).These studies revealed that the major PB-inducible gene family comprises two genes which are more than 97% homologous at the DNA level (Suwa et al (1985)). Both are highly inducible by PB, but the product of one (PB_{3a}) appears to have much higher enzymic activity towards a number of substrates than that of the other (PB_{3b}) (Ryan et al (1982)). The role of the small differences

between these genes in determining the differing enzymic activity of their products appears to involve the environment surrounding the haem moiety of the enzyme (Wolf et al (1988)); further studies on these genes may help to localise the parts of the protein which are involved in substrate binding and catalysis.

Intensive studies on PB-induction have been carried out over a number of years, but the mechanism by which PB regulates P-450 expression is still poorly understood. Treatment of experimental animals with PB stimulates the de novo synthesis of P-450 mRNA, leading to an increased rate of P-450 protein synthesis (Adesnik et al (1981), Adesnik and Atchison (1985) Pike et al (1985)). Hybridisation of nascent mRNA transcripts indicates that an increase in transcription rate occurs within 30 minutes of PB treatment, reaching a peak of 25 - 50 times the constitutive rate after 4 hours and returning to its control rate after 38 hours (Adesnik and Atchison (1985)). The mechanism of this dramatic increase in transcription rate is not understood: PB treatment causes a general increase in transcriptional activity in the liver (Hardwick et al (1983a)), and the existence of a "PB-receptor" has been postulated, but many years of intensive research have failed to identify any such entity. A number of observations argue against the existence of a specific PB-receptor: high doses of PB are required in order to induce P-450 expression, and the many PB-like inducing agents do not share any readily discernable structural homology (Snyder and Remmer (1979)), but if a receptor does not exist it is difficult to see how specific induction of certain genes and not others might be mediated. The search for the hypothetical PB-receptor continues: advances in molecular biology have opened up a number of new possibilities such as the use of DNA footprinting, identification of DNAase hypersensitive sites and the use of hybrid genes to study the mechanism of P-450 regulation by PB. One problem which remains is the shortage of PB-responsive cell lines in which the regulation of P-450 expression by PB could be studied by making hybrids between the 5' domain of a PB-inducible P-450 gene and the chloramphenicol acetyl transferase gene and examining the regulation of this hybrid gene by PB.

As well as regulation by xenobiotics, an interesting aspect of the regulation of PB-inducible P-450s is tissue-specific expression: a high levels of PB_{3a} is expressed in rabbit lung (Wolf et al (1980)) whereas in the liver the level of expression is low prior to treatment with inducing agents. Little is known about this aspect of P-450 regulation: it is assumed that trans-acting factors such as hormones

are involved, but their nature has not yet been established. A further complication is the fact that although the lung expresses high levels of PB_{3a}, further induction by PB does not occur in this organ. This may indicate either (a) that the mechanism by which this gene is induced in the liver is permanently switched on in the lung, or (b) that a completely different mechanism of regulation operates in the lung. It thus appears that a heirarchical system of regulatory factors may exist in which binding of trans-acting factors, possibly including a lung-specific factor and a PB-receptor (or its second messenger) to cis-acting regulatory elements of the PB_{3a} and PB_{3b} genes regulates the rate of transcription of these genes; in such a system, the rate of transcription of PB-inducible genes at a given time would depend on the contribution of each member of this regulatory heirarchy (Whitlock (1986)).

1.3.3. The P450III gene family: Glucocorticoid-inducible P-450s.

In the 1970s it was found that catatoxic steroids conferred protection against the toxic effects of certain chemicals by increasing the rate of their metabolism (Selye (1971). The most potent of these steroids was pregnenolone-16α-carbonitrile (PCN), which was found to be one of a new class of inducing agents (Lu et al (1972)). In 1980, the first example of a PCN-inducible P-450 was purified from rat liver (Elshourbagy and Guzelian (1980)). This enzyme had a number of novel biochemical, immunological and catalytic properties which implied that it represented a previously unidentified type of P-450, and this gene has now been assigned to P-450 Family III (Nebert et al (1987)). The full spectrum of activity of this isozyme is not known, although there is some evidence that it is responsible for the oxidation of the drug nifedipine. The mechanism of induction of this isozyme by PCN and other steroids is incompletely understood even though the glucocorticoid receptor system has been well characterised (Rousseau (1984)). A sixfold increase in the level of the mRNA encoding this protein occurs in male rat liver within 3 hours of PCN treatment (Hardwick et al (1983b)), but studies using primary rat hepatocyte culture suggest that, unlike the regulation of "classical" glucocorticoid-responsive genes such as tyrosine aminotransferase, induction of P-450s by glucocorticoids does not appear to proceed via a classical receptor-mediated mechanism. It is unclear whether induction of P-450 is mediated by an atypical glucocorticoid receptor or proceeds via a non receptor-mediated mechanism. In more recent studies the effects of other inducing agents on this isozyme have been studied (Schuetz et al (1986a,b)), and this has led to the finding that induction may occur both at the level of de novo synthesis (eg. by Dex) and by specific

inhibition of protein degradation (eg. by the antibiotic triacetyloleandomycin) (Watkins et al (1986)). PB also induces the synthesis of this isozyme (Guengerich et al (1982a)): this project will refer to this isozyme as PB_{2C}, since the preparation used for antibody production was purified from PB-induced rat liver.

1.3.4. The P450IV gene family: Peroxisome proliferator-inducible P-450s.

The hypolipidemic peroxisome proliferators, including clofibrate, represent a novel class of hepatocellular tumour promoters (and are non-genotoxic carcinogens) in rodents (Reddy et al (1980)). They also induce a P-450, called LAω or P-452, having high specificity towards lauric acid ω-hydroxylation in rat liver (Parker and Orton (1980)). The induced P-450 protein exhibits a complete lack of immunological cross-reactivity with P-450s induced by the known classes of inducing agents (Tamburini et al (1984)) and has been assigned to family IV, of which it is the only known member. A full length cDNA encoding this P-450 has been characterised; its amino acid sequence is less than 33% homologous to those of members of the other mammalian P-450 families (Hardwick et al (1987)). Little is known about the mechanism of regulation of this P-450: it is possible that the mechanisms of peroxisome proliferation and P-452 induction are linked. If this is the case, the question of the species specificity of induction is intriguing; certain systems, including human hepatocytes, are unresponsive to the peroxisome proliferating effects of chemicals such as clofibrate (C.R.Elcombe, personal communication). It would be interesting to discover whether any of these systems is responsive to induction of P-452 by these agents, and whether P-452 induction or peroxisome proliferation is the mechanism of their carcinogenic effects (Popp et al (1989)).

1.4. Regulation of P-450-dependent drug metabolism during infection and inflammation.

Exogenous inducing agents can, as described above, cause dramatic changes in P-450-dependent drug metabolism; however, endogenous regulation of P-450s is also important in determining hepatic drug-metabolising capacity in health and disease. Microsomal drug metabolism is affected by a number of abnormal physiological states such as starvation, liver, heart or kidney disease, hormonal disturbances, presence of a tumour, adjuvant arthritis, changes in the reticuloendothelial system and other factors including stress, irradiation and heavy metals (Kato (1977)). Parasites,

bacteria and viruses also cause inflammation leading to changes in P-450-dependent drug metabolism. Studies in human populations have shown that such changes can affect the clearance of a variety of drugs; this is particularly important in the case of drugs such as warfarin and theophylline which have a low therapeutic index.

1.4.1. Effects of disease states on human drug metabolism.

Clinical data shows that human drug metabolism is impaired during viral and bacterial infections. In several studies the half-life of theophylline was extended during influenza infection, leading to toxic effects in previously well-controlled asthmatics (Chang et al (1979), Kraemer et al (1982)). Administration of theophylline following influenza vaccination of healthy volunteers, asthmatics and patients recovering from chronic obstructive pulmonary disease confirmed this finding (Renton et al (1980), Walker et al (1981)); similar results were obtained in studies using aminopyrine and warfarin (Kramer et al (1981)). Administration of recombinant interferon also suppressed antipyrine and theophylline metabolism in human volunteers, leading to the suggestion that interferon-inducing impurities in influenza vaccine were responsible for its P-450-suppressive effect (Williams and Farrell (1986), Williams et al (1987), Winstanley et al (1987)). The effects of other infectious diseases on human drug metabolising capacity have been studied in less detail, with conflicting results. Infection with the bacterium Haemophilus influenzae appeared to suppress theophylline clearance in some cases (Renton (1983)) whereas in an epileptic suffering from infectious mononucleosis, phenytoin clearance was accelerated (Leppik et al (1979)). Clarification of the effects of infection on the clearance of specific drugs is needed, but it is clear that infectious diseases have a significant effect on human hepatic drug metabolism.

Studies of patients suffering from acute uncomplicated viral hepatitis showed that liver disease also affected drug clearance (Kato (1977)). In most cases the clearance of drugs which are metabolised via P-450-dependent routes was impaired; for example, pentobarbital hydroxylation was decreased in a group of 17 non-drug addicts showing symptoms of acute viral hepatitis (Doshi et al (1972)). Chronic liver disease has similar effects, the clearance of many drugs being impaired (Schoene et al (1972)). Table 1.2 summarises a number of studies: it will be noted that several drugs appear in more than one section of the table, reflecting contradictions regarding the effects of liver disease on their clearance. The problems experienced may be related to the fact that patients with liver disease are often treated with drugs which

alter P-450 levels, with different pathways being altered to differing extents. Binding of drugs to albumin is also affected by liver disease, and this may be a factor in the altered clearance of some drugs.

Table 1.2.

Effects of liver disease on drug clearance in humans.

Effect on clearance	Drug	Disease state
Increased half-life	Hexobarbital	Acute viral hepatitis
	B-methyl digoxin	Acute viral hepatitis
	Meperidine	Acute viral hepatitis, cirrhosis
	Diazepam	Acute viral hepatitis, cirrhosis
		Chronic active hepatitis
	Tolbutamide	Acute viral hepatitis, cirrhosis
		Chronic liver disease
	Meprobamate	Chronic hepatitis, cirrhosis
	Amobarbital	Cirrhosis
	Aminopyrine	Laennec's or portal cirrhosis
	Antipyrine	Laennec's cirrhosis
	Glutethimide	Chronic liver disease
	Lidocaine	Chronic liver disease
	Diphenylhydantoin	Liver disease
	Phenobarbital	Liver disease
No change in half-life	Diphenyl hydantoin	Acute viral hepatitis
	Phenobarbital	Acute viral hepatitis, cirrhosis
	Aminopyrine	Laennec's or portal cirrhosis
	Salicylic acid	Laennec's cirrhosis
	Dicumarol	Laennec's cirrhosis
	Antipyrine	Laennec's cirrhosis
	Phenylbutazone	Laennec's cirrhosis, liver disease
	Tolbutamide	Chronic liver disease, cirrhosis
	Pentobarbital	Severe liver disease
	Acetanilide	Chronic liver disease
Decreased half-life	Phenylbutazone	Cirrhosis

Summarised from Kato (1977).

1.4.2. Effects of parasites, bacteria and viruses on hepatic P-450 function in experimental animals.

The effects of infection on hepatic P-450 function have been widely studied in laboratory animals. These studies have significant implications for humans and

domestic animals treated with drugs which are metabolised via the P-450 system.

Several types of parasites suppress hepatic drug metabolism. These include the malarial parasite Plasmodium berghei (McCarthy et al (1970)), the helminth Fasciola hepatica (Maffei-Facino et al (1981)), the liver fluke Schistosoma mansoni (Cha and Edwards (1976)) and the agent of sleeping sickness, Brucei gambiense (Shertzer et al (1981)). In these studies, general parameters of drug metabolism including aniline, nitroanisole, ethylmorphine, hexobarbital, zoxazolamine and B(a)P metabolism were measured, making it difficult to identify the P-450 isozymes affected. However, in one recent study (Galtier et al (1986)) Western blot analysis was used to show that isozyme UT-A (a constitutive form) was strongly suppressed by infection with F. hepatica whereas the major PB- and 3-MC-inducible forms were not significantly affected.

The mechanism of P-450 suppression by parasites has received little consideration to date; the most complete studies have been those involving S. mansoni. Although these studies were dogged by technical problems (Cha and Edwards (1976), El-Mouelhi et al (1987)), the use of different strains of mice and patterns of infection made it possible to determine the physiological mechanism involved. Two facts, the failure of unisexual infections to suppress P-450 levels (Cha et al (1980a)) and the resistance of congenitally athymic nu/nu mice, which cannot mount a T-cell dependent granulomatous response to S. mansoni eggs, to P-450 suppression by mixed infections (Cha et al (1980b)) implicated the parasite eggs, and specifically the host's inflammatory response thereto, in this effect.

Several types of virus have been shown to alter hepatic P-450 levels. In early studies, mouse hepatitis strains MHV-B and MHV-3 caused about 50% suppression of hepatic P-450 and metabolism of hexobarbital, strychnine, pentobarbital and aniline in Swiss albino mice (Kato et al (1963), Budillon et al (1972)). The PB-induced levels of these enzymes were less susceptible than the uninduced levels. The effects of hepatitis virus could have been due to primary pathological changes in the liver; however, other viruses which did not cause such changes also suppressed hepatic P-450-dependent drug metabolism. These included encephalomyocarditis virus (Renton (1981a)), Mengo virus (Renton and Mannnering (1976)) and Newcastle Disease virus (Singh and Renton (1981)). Suppression of hepatic P-450 was not, however, common to all viruses; in experiments using BALB/c and CD-1 mice,

influenza virus infection significantly suppressed pulmonary B(a)P hydroxylase activity (90% suppression 10 days after infection) but had little effect on hepatic B(a)P hydroxylase or ethylmorphine N-demethylase, and only a transient effect on 7-EC deethylase (Corbett and Nettesheim (1973), Rabovsky et al (1986)).

The common effect of these infective agents is to activate host defence mechanisms. Mannering and his colleagues proposed that such mechanisms, especially interferon induction, may be responsible for P-450 suppression. This hypothesis is supported by experiments using other agents which activate host defence mechanisms.

1.4.3. Role of Host Defence Mechanisms in P-450 regulation.

Studies using compounds which activate host defence mechanisms indicate that the reticuloendothelial system and inflammatory responses are important modulators of P-450-dependent drug metabolism in the liver. Administration of non-specific immunostimulants usually resulted in a decrease in hepatic microsomal drug metabolism and the capacity of the liver to eliminate drugs.

Three commonly studied agents are Corynebacterium parvum, Bordetella pertussis and Bacillus Calmette Guerin (BCG), which have been used as immunoadjuvants in cancer chemotherapy. Treatment of mice with C. parvum increased sensitivity to pentobarbital, reduced aminopyrine, aniline and p-nitroanisole metabolism, and suppressed P-450 levels in the C57BL/6J mouse and the Sprague-Dawley rat (Soyka et al (1976), Farquhar et al (1983)). The effect was time-dependent, with maximum suppression occuring six days after C. parvum treatment and recovery around day 17, but was not dose-dependent, especially in the C57BL/6J mouse. A number of enzyme activities (aminopyrine N-demethylase, ethylmorphine N-demethylase and aniline hydroxylase) and total P-450 levels were suppressed 30 - 40% by treatment of CFW or CD-1 mice with 7×10^9 killed B. pertussis cells (Williams and Szentivanyi (1977)); the half-life of phenytoin in Sprague-Dawley rats was also extended by this agent (Renton (1979)). Similarly, intravenous injection of BCG organisms to Sprague-Dawley rats suppressed aniline hydroxylase, 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide demethylase and total P-450, but subcutaneous or intradermal administration did not have this effect, implying that systemic BCG was required (Farquhar et al (1976)).

Little is yet known about the mechanism by which immunoadjuvants suppress

P-450-dependent drug metabolism in the liver; the agents may act via different mechanisms since C. parvum appears to exert its effects via macrophage-mediated responses, whereas B. pertussis also affects T-cells (Renton (1983)). In male CBA/6J mice, the effects of C. parvum on P-450 expression were prevented by splenectomy, whole body irradiation and compounds which block macrophage activity (Soyka et al (1979)). These data implicated macrophages in the response, although a deeper understanding of the relative roles of T- and B- lymphocytes is needed for further elucidation of the mechanism. Activated macrophages may also be involved in the effects of BCG on P-450s (Farquhar et al (1976)) although little experimental data has been presented to support this proposal. Another possible mechanism of action of C. parvum and BCG involves induction of the haem-degrading enzyme haem oxygenase; both agents induce this enzyme, but no causal relationship with P-450 suppression was established (Matsuura et al (1985)). Attempts were made to identify the cellular components of B pertussis involved in P-450 suppression. It appeared that two components were involved: one, histamine sensitising factor, which is labile to 80^{o}C heat, caused the short term ($\leq$ 24 hours) suppression of P-450 whereas a second, non-heat labile component, possibly endotoxin, was associated with long-term effects (Williams et al (1980)).

Another model used to elucidate the effects of inflammation on P-450-dependent activities is adjuvant-induced arthritis, induced by injection into the rat footpad of organisms such as Mycobacterium butyricum or Mycobacterium tuberculosis in parrafin oil: the disease develops within about 15 days of the injection (Renton (1983)). During adjuvant-induced arthritis, a variety of P-450-dependent functions were suppressed, including N-demethylase activity, suppressed by 15% five days after adjuvant treatment and by as much as 93% by day 15, cyclophosphamide activation and clearance of hexobarbital, zoxazolamine and barbital (Morton and Chatfield (1970), Beck and Whitehouse (1973), Sofia (1977)). Total P-450 levels were correspondingly suppressed during adjuvant arthritis and P-450 was converted to its degradation product, P-420 (Mathur et al (1977)). No defects in tryptophan oxidase and 5-aminolevulinic acid (ALA) synthetase able to account for the loss of P-450 in adjuvant arthritic rats were detected (Cawthorne et al (1976)). It was the arthritic response, rather than the presence of infective agent per se, which appeared to cause P-450 suppression: effects on drug metabolism are not observed if the tubercle bacilli are injected directly into a lymph node (Morton and Chatfield (1970)). The effects of adjuvant arthritis on hepatic drug metabolism were to some

extent reversed by treatment with anti-inflammatory drugs such as dexamethasone, corticosterone or azothiaprine, but immunosuppressive agents (cyclophosphamide, cycloleucine or azauridine triacetate) and non-steroidal anti-inflammatory drugs had little or no effect on this phenomenon (Beck and Whitehouse (1974)).

A few studies have assessed the effects of P-450 inducers on the suppression of drug metabolism by adjuvant arthritis; these concentrated on hoped-for benefits in terms of reducing the disease's severity, rather than addressing the mechanism of P-450 suppression. In a 1975 study, B(a)P gave some protection against suppression of AHH activity (Carlson and Ciaccio (1975)), but the activity was measured 9 - 16 days after B(a)P administration, by which time it had almost returned to its control value making it hard to assess the significance of the finding in terms of P-450 induction versus suppression. Similarly, PB protected rats against loss of aniline hydroxylase, aminopyrine N-demethylase and p-nitroanisole N-demethylase activities and abolished the appearance of P-420 relative to uninduced controls (Mathur et al (1977)), but the activities observed in animals treated with PB plus adjuvant were much lower than those in animals given PB alone. Although it prevented loss of P-450-dependent enzymes, PB was unable to ameliorate the course of the disease, indicating that loss of P-450 is not involved in the development of arthritic symptoms.

These studies showed that the reticuloendothelial system of the liver, which comprises a number of cell types including Kupffer cells, the resident macrophage population of the liver, was important in the regulation of drug-metabolism. Others showed that steady-state drug-metabolism was reduced if increases or decreases in the reticuloendothelial system occurred (Wooles and Munsen (1971)). When Kupffer cells were loaded with carbon particles, the metabolism and hepatotoxicity of carbon tetrachloride was reduced, as were hepatic levels of P-450, cytochrome b_5 and ethylmorphine N-demethylase activity (Stenger et al (1969), Leterrier et al (1973)). Other reticuloendothelial system activators, including maleic anhydride ethers, immunoadjuvant peptidoglycan, high molecular weight dextrans and small latex beads had similar effects (Barnes et al (1979), Tresec et al (1983) Williams and Szentivanyi (1983), Peterson and Renton (1984, 1986)). These factors have multiple hepatic effects including reticuloendothelial system blockage, immunomodulation and interferon induction, some of which are mediated by

lymphokines; an understanding of the role of these agents is important to comprehension of the effects of reticuloendothelial system activators on P-450.

1.4.4. Effects of inflammatory mediators on hepatic P-450 levels.

The studies described above indicated that inflammation and activation of the immune system are important in determining the drug metabolising status of the liver both in humans and animals; however, the systems studied were too complex to give useful information about the mechanisms of the observed effects. Further studies tried to identify more specific agents which might be involved, beginning with such relatively non-specific factors as endotoxin and progressing to the study of purified, and later recombinant DNA-derived, mediators such as interferons.

Endotoxin (lipopolysaccharide) is a toxic cell wall component of gram negative bacteria comprising O-specific polysaccharide, core polysaccharide and lipid A. It is present in large quantities in the intestine as a result of bacterial death and release during active growth. Small amounts of endotoxin are regularly absorbed, but are rapidly detoxified by the liver, which acts as a barrier to bacterial toxins arising in the gut (Nolan (1981)). Kupffer cells, fixed macrophages first described by von Kupffer in 1876, are the major site of endotoxin detoxification. If stimulated by endotoxin, Kupffer cells release inflammatory mediators and enzymes including lysozyme, collagenase, interleukin-1 and prostaglandins (Nolan and Camera (1982)). In a study using female Holtzman rats, aniline hydroxylase, B(a)P hydroxylase and P-450 levels were significantly suppressed by a 1mg dose of E. coli endotoxin. This effect did not correlate with hepatocyte damage (Gorodischer et al (1976)). Endotoxin released during intestinal ulceration was also implicated in suppression of P-450-dependent activities by indomethacin (Burke et al (1983), Falzon et al (1984)), although endotoxin was not the only factor involved in the suppressive effects of indomethacin, since the two agents did not have identical effects. The mechanism of P-450 suppression by endotoxin is not yet understood. Endotoxin was long ago shown to induce the haem-degrading enzyme haem oxygenase in macrophages, sinusoidal cells and hepatocytes (Gemsa et al (1974)); however, since the mechanism of this effect is itself unclear this observation is insufficient to elucidate the mechanism of P-450 suppression. Endotoxin has many other effects, including reticuloendothelial system activation, adjuvant-like effects and induction of interferon and other lymphokines such as interleukin-1 and tumour necrosis factor; one of these may be responsible for the observed suppression of drug metabolism.

A variety of other interferon inducers have also been used to study the effects of host defence mechanisms on P-450-associated activities. Initially it was observed that tilorone (2,7-bis[2-diethylaminoethoxy]fluoren-9-one) suppressed P-450 and its dependent activities, including ethylmorphine N-demethylase, aminopyrine N-demethylase, aniline-p-hydroxylase and hexobarbital oxidase (Leeson et al (1976), Renton and Mannering (1976)); this led to studies on a variety of other interferon inducers, all of which were found to have similar effects (Renton and Mannering (1976), summarised in Table 1.3). Induction of interferon was proposed to be the mechanism involved, but all the compounds tested have other effects, including immune modulation and activation of host defence mechanisms, which might also participate in P-450 suppression (Renton (1981)).

Circumstantial evidence indicated that the effects of interferon inducers on P-450 expression really were mediated by interferon. Neither polyriboinosinic acid (poly I (10mg/kg)) nor polyribocytidilic acid (poly C (10mg/kg)) induces interferon, nor do they suppress P-450. However, when an injection of poly I (5mg/kg) was followed an hour later with an injection of poly C (5mg/kg), as much interferon was induced as by polyriboinosinic acid-polyribocytidylic acid (poly IC) and drug metabolism was depressed. When the order of injection of poly I and poly C was reversed, neither interferon induction nor depression of the P-450 system was observed (Deloria and Mannering (1982)). Inbred strains of mice which differ in their response to interferon induction by Newcastle disease virus were also used to relate P-450 suppression to interferon levels. Poly IC induces approximately equal serum interferon titres in C57BL/6J and C3H/HeJ mice, but Newcastle disease virus induces only about one-tenth as much serum interferon in C3H/HeJ mice as in C57BL/6J mice. A 5×10^7 pfu dose of Newcastle disease virus suppressed P-450s in C57BL/6J mice but not C3H/HeJ mice whereas poly IC (10mg/kg) suppressed P-450 in both strains; however, the effect of injecting interferon directly into the C3H/HeJ mice was not tested in this study, so suppression of P-450 by a non-interferon-mediated mechanism was not excluded (Singh and Renton (1981)). Studies using the F_1 hybrid of these strains, the B6C3F_1 mouse, would confirm these results as well as being interesting because this hybrid is used in regulatory toxicology in the USA.

Table 1.3.

Interferon inducers which suppress P-450 expression in male Holtzman rats.

Interferon inducer	Nature of substance	Dose administered	P-450 (% control)
Mengo virus	RNA virus	5×10^7pfu/kg	77.1± 4.5
Statolon	Fungal mycophage	50mg/kg	59.4 ± 1.8
Hepatic RNA	Polyribonucleotide	5mg/kg	69.5 ± 2.7
Poly IC	Double stranded RNA	2.5mg/kg	64.8 ± 5.1
E.coli endotoxin	Cell wall component	5mg/kg	54.9 ± 5.3
B. pertussis vaccine	Immunoadjuvant	2×10^{11}cells/kg	77.0 ± 6.0
Quinacrine	Antimalarial drug	50mg/kg	62.0 ± 7.0
CP 20,901	N,N-dioctadecyl-n'n'-bis(2-hydroxyethyl) propanediamine	50mg/kg	44.8 ± 6.1
Tilorone	2,7-bis[2-diethyl aminoethoxy] fluoren-9-one	50mg/kg	64.5 ± 5.8
RMI 11002	3,6-bis(dimethyl aminoacetyl)fluorene	50mg/kg	55.7 ± 5.9
RMI 11567	3,6-bis(dimethyl aminoacetyl) dibenzofuran	50mg/kg	72.0 ± 8.7
RMI 11877	3,6-bis(dimethyl aminoacetyl) dibenzothiophene	50mg/kg	49.4 ± 7.6

Summarised from Renton and Mannering (1976).

The above studies concerned the effects of compounds which induce interferons (ifns) α and β. Recently, a number of studies considered the effect of ifn γ on P-450.

Endogenous ifn γ was induced by injection of 50mg of tuberculin into mice sensitised three weeks previously with BCG, with a "mock interferon" control consisting of mice treated with tuberculin without prior sensitisation. The results indicated that whilst BCG suppressed P-450 levels and metabolism of aminopyrine and diphenylhydantoin, this was potentiated by subsequent injection with tuberculin, an effect attributed to the induction of ifn γ. No P-450 suppression was detected in the "mock interferon" control, and the degree of suppression correlated with the titre of interferon induced (Sonnenfeld et al (1980), Harned et al (1982)). Similar results were obtained when a "semi-purified" preparation of ifn γ was injected into mice (Sonnenfeld et al (1982), Smith et al (1983)). Ames tests using S9 mix from mice in which ifn γ has been induced by the above protocol, showed that the metabolic activation of the carcinogens 2-acetylaminofluorene, aflatoxin B_1 and B(a)P is also significantly suppressed (Reiners et al (1984)). The extent of suppression was around 50% in the cases of 2-acetylaminofluorene and aflatoxin B_1 but was more than 95% in the case of B(a)P. This result is important in that it indicates a significant effect of ifn γ on the mutagenicity, and by implication the carcinogenicity, of compounds which undergo P-450-dependent activation.

Proof of the involvement of interferon in the depression of hepatic P-450 awaited the availability of a pure interferon that was active in a laboratory animal. This came in the form of hybrid HuIFNr-AD, which possessed antiviral activity in the mouse. HuIFNr- AD depressed the P-450 system of the mouse; HuIFNr-A and HuIFNr-D, which have no antiviral activity in the mouse, had little or no effect. Pure recombinant mouse ifn γ also depressed the P-450 system, possibly by a mechanism different from that initiated by ifns α and β (Mannering and Deloria (1986)). Recent reports concerning the effects of recombinant interferons and other lymphokines on P-450 expression are discussed in Chapter 5.

The mechanism of depression of the P-450 system by interferon remains obscure: conflicting results arose from studies attempting to distinguish suppression of de novo P-450 synthesis from increased haem degradation. Poly IC and tilorone affect a number of enzymes which are involved in haem homeostasis; events taking place during the suppression of P-450 have been interpreted as indicating that they perturb the haem pool by increasing the rate of dissociation of haem from P-450 or by

inhibiting the de novo synthesis of P-450 protein, leading to elevation of tryptophan-2,3-dioxygenase, depression of ALA-synthetase and induction of haem oxygenase. By decreasing the rate of haem synthesis and increasing degradation the cell disposes of the excess haem released from P-450 (El Azhary and Mannering (1979)). This group also used an isotopic labelling method to assess the metabolism of haem in Sprague-Dawley rat liver, concluding that the rate of degradation of P-450 increased in response to poly IC (El Azhary et al (1980)). However, the technique used (measurement of the incorporation of ALA-3,5-^{3}H into haem) did not specifically consider P-450 haem as opposed to the rest of the haem pool, so this result is inconclusive. No evidence was found for a decrease in P-450 synthesis, but a later paper reported both an increase in haem degradation and a decrease in P-450 protein synthesis in C57BL/6J mice treated with poly IC (10mg/kg) (Singh and Renton (1984)). Again, a non-specific technique was used (gel filtration analysis of radiolabelled proteins), and it is, of course, possible that the response of rat and mouse liver to poly IC differs, but these results illustrate the prevailing confusion regarding the level at which P-450 suppression occurs as well as its mechanism. Similarly, a study using a dual isotope method to determine the rates of synthesis and degradation of microsomal proteins indicated that "treatment of mice with poly IC inhibits protein synthesis and stimulates protein degradation. The effects of this are seen primarily in the endoplasmic reticulum because it is the most rapidly turning over organelle of the mouse liver" (Gooderham and Mannering (1986)). Like the other studies, this experiment was unable to distinguish effects on P-450s from those on other microsomal proteins, so that it is uninformative concerning the specific effects of interferon on P-450s.

More specific studies indicated that all P-450 isozymes are not equally suppressed by interferon or interferon inducers. Comparison of the effects of tilorone, poly IC, and adjuvant treatment on protein expression in male albino rat liver by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence scanning indicated that several proteins in the molecular weight range 43,000 - 54,000 were significantly suppressed. Different treatments led to the suppression of different proteins, suggesting that the inducers might be acting via alternate mechanisms (Zerkle et al (1980)). The proteins affected were not shown to be P-450s: the technique of Western blot analysis, which could have identified the proteins examined, was just becoming available and was not used.

The studies discussed above show that hepatic drug metabolism may be dramatically affected by infection and inflammation; the aim of this project is to identify a model in which to study these aspects of P-450 modulation, in the hope of identifying the endogenous agents involved. Previous studies concentrated on the liver because this is quantitatively the major organ involved in drug metabolism. However, other organs also have the capacity to perform P-450-dependent drug metabolism; these include the lung and the colon, tissues which are of particular interest both because they are susceptible to inflammation and because tumours in these organs are notoriously difficult to treat by conventional therapy. A better understanding of the relationships between P-450 regulation, inflammation and cancer in these organs might offer new possibilities for treatment of such refractory tumours.

1.5. Xenobiotic metabolism and inflammation in the lung and colon.

It is clear that modulation of hepatic P-450 activity by xenobiotics and inflammatory mediators is an important determinant of responsiveness to drugs and carcinogens in both humans and experimental animals. Two other organs which are exposed to both xenobiotics and inflammatory mediators are the lung and the colon. In the lung, smoking results in exposure to carcinogens as well as leading to the development of inflammatory diseases including emphysema and chronic bronchitis. The colon is continuously exposed to dietary carcinogens, and colon cancer is linked with inflammatory colonic diseases in a small but significant number of cases.

1.5.1. P-450-dependent xenobiotic metabolism in the lung.

The structure of the lung is well suited to the clearance of air- and blood-borne chemicals; venous blood from the whole body perfuses through the alveolar- capillary unit, whilst the thin epithelial and endothelial layers of the alveolus facilitate gaseous exchange between pulmonary blood and the atmosphere. In modern cities, the lung is continuously exposed to airborne carcinogens such as B(a)P, which is formed in various ways in the human environment (Badger et al (1960)); levels as high as 40$\mu g/m^3$ have been reported in polluted air (Doll and Peto (1981)). An important source of airborne carcinogens is tobacco smoke: it has long been known that both cigarette and pipe smoke contain high levels of PAHs (Commins et al (1954), Gilbert et al (1956)), and tobacco is the direct cause of about one third of all cancer deaths (Peto (1981)), but smokers and their social contacts are still regularly exposed to potent carcinogens found in tobacco smoke.

The total P-450 level of uninduced lung is lower than that of liver, but pulmonary clearance of xenobiotics can, in some circumstances, be as important as hepatic clearance; for example, in 3-MC-treated rats pulmonary and hepatic clearance of B(a)P are almost equally efficient (Bend et al (1985)). Information about P-450 expression in specific lung cell types was derived from studies using isolated cell populations, which indicated that pulmonary endothelium contains a heterogeneous distribution of cell types expressing differing levels of P-450s (Devereux (1984)). Autoradiographic studies with the lung-specific toxin 4-ipomeanol first showed that the Clara cell was a major site of lung P-450-dependent mixed function oxidase activity (Boyd (1977)); the alveolar Type II pneumocyte, the alveolar macrophage and the bronchial epithelial cell also express P-450s (Baron et al (1988)). Both the Clara cell and the alveolar Type II cell are capable of metabolising P-450 substrates including 7-ethoxycoumarin (7-EC), coumarin and B(a)P (Devereux (1984)) and are targets for toxic chemicals such as 4-ipomeanol and carbon tetrachloride (Boyd et al (1980) Boyd (1982), Devereux et al (1982)).

The profile of P-450s expressed by the lung differs considerably from that of the liver. Differences in susceptibility to xenobiotics between the liver and the lung are probably a function of the proportions of P-450 isozymes expressed and their regulation, since the lung has the capacity to express the same P-450 proteins as the liver (Bend and Serabjit-Singh (1984)). In the uninduced rabbit lung the major isozymes expressed are Isozyme 2 (PB_{3a}) and Isozyme 5 (related to PB_1) (Wolf et al (1980), Vanderslice et al (1987)). These are minor components of the liver P-450 system except in animals treated with PB, whereas PB causes hardly any P-450 induction in the lung (Philpot and Smith (1984)). The high level of these isozymes enables the lung to efficiently metabolise benzphetamine, ethylmorphine, aminopyrene and p-nitroanisole. Lung microsomes are also 20 - 30 times more effective at metabolising the aromatic amines 2-aminofluorene and 2-acetylaminofluorene than liver microsomes; Isozyme 5 is very active towards this class of carcinogens (Robertson et al(1981), Vanderslice et al (1987)).

The uninduced rabbit lung expresses detectable concentrations of Isozyme 6 (MC_{1b}); as in the liver this isozyme is further induced by Ah ligands. The relatively high level of Isozyme 6 in the lung compared with the uninduced liver is thought to be an important factor in pulmonary susceptibility to PAH carcinogenesis (Philpot and

Smith (1984)). In rabbits treated in vivo with TCDD a 20 fold increase in the pulmonary expression of MC_{1b} has been detected by Western blot analysis (Domin and Philpot (1986)). Both Clara and Type II cells, which already express detectable levels of MC_{1b} prior to induction, showed this increase in expression following TCDD treatment. It was interesting that the alveolar macrophage fraction exhibited a dramatic (90-fold) increase in expression from negligible levels in the uninduced state to significant levels following induction, indicating that in the induced state the alveolar macrophage has the capacity to play a significant role in the metabolism of inhaled PAHs (Domin et al (1986)). As in the liver, pulmonary induction of MC_{1b} is mediated by the Ah receptor, which is highly concentrated in the lung relative to other extrahepatic organs (Mason and Okey (1982)). In a comparison of pulmonary B(a)P metabolism in various strains of mice, the lungs of genetically "Ah-responsive" A/HeJ mice responded to 3-MC treatment with a 50 fold increase in B(a)P metabolism; in the lungs of C57BL/6 mice 20 fold induction was observed. Even in the lungs of "unresponsive" DBA/2J mice 20 fold induction of this activity was observed, although the absolute activities observed were lower and the profile of metabolites differed from those of the responsive strains (Seifried et al (1977)). The reason for this difference in Ah-responsiveness between mouse lung and liver has not been elucidated.

Although pulmonary P-450-dependent metabolism has been extensively studied in experimental animals, particularly in the rabbit, relatively few studies have been performed using human material, mainly because of the difficulty of obtaining fresh samples with which to work. It has, however, been shown that cigarette smoke can induce P-450s in human lung (Cantrell et al (1983)) as well as liver (Thompson et al (1984), Pelkonen et al (1986)). Recently, more detailed studies on P-450 induction in human lung have been carried out: de Flora et al (1987), for example, carried out an extensive study which showed that AHH activity was significantly lower in lung tumours than in normal lung parenchyma. It was interesting that no correlation between AHH levels and the number of cigarettes smoked was found in this study. The same group also found that although there was no clear relationship between lung AHH activity and number of cigarettes smoked, lungs of recent smokers did have elevated AHH activity compared with those who had ceased smoking more than thirty days before resection (Petruzzelli et al (1988)).

The most detailed studies on carcinogen metabolism in human lung material have been

performed using lung explants in short-term culture, with B(a)P as a model carcinogen. The activation of B(a)P is discussed in detail in Chapter 4; briefly, the primary metabolites of B(a)P are the (4,5), (7,8) and (9,10)-epoxides. These are further metabolised to form diol epoxides which are direct mutagens. The most active of these diol epoxides is the 7,8-diol-9,10-epoxide, and the pathway proposed as the major route of mutagenic activation is:

	P-450	EH	P-450
B(a)P	-> B(a)P-7,8-oxide	-> B(a)P-7,8-diol	-> B(a)P-7,8-diol-9,10-epoxide.

Early studies on the formation of B(a)P-DNA adducts in human bronchial explants showed that the process was dependent on time, temperature and B(a)P concentration; use of the inhibitor 7,8-benzoflavone indicated that it was mediated by P-450 (Harris et al (1976). High performance liquid chromatographic analysis showed that the adducts formed were indeed derived from B(a)P diol epoxides(Harris et al (1977)). Similar experiments using explants of peripheral lung and monolayer cultures derived from human lung tumours helped to confirm these findings (Shinohara and Cerutti (1977), Siegfried et al (1986)). Further work showed that there is large interindividual variability in the extent of B(a)P-DNA adduct formation by human bronchial explants, although at first no significant difference between lung cancer and non-lung cancer patients was detected (Harris et al (1977); however, in studies using larger subject populations a relationship between carcinogen activation and tumour type was observed; there was no significant difference in carcinogen activation between non-cancer samples and samples from patients bearing well differentiated adenocarcinomas, but patients bearing primary epidermoid tumours had elevated B(a)P metabolising capacity. A family history of lung cancer was associated with increased B(a)P activation by lung explants (Harris et al (1984)). It appears that pulmonary P-450s are important determinants of susceptibility to lung cancer, and our present incomplete understanding of pumonary P-450 regulation should certainly be extended. A group of conditions which have a dramatic effect on the metabolic status of the lung, those involving inflammation, are among those most likely to affect pulmonary P-450-dependent drug metabolism.

1.5.2. Inflammation and cancer of the lung.

The need for efficient air exchange between the lung and its surroundings makes it vulnerable to damage by infection and pollutants inhaled in the air. The lung defends

itself against infection and particulate pollutants by a number of mechanisms including continuous upward flow of mucus in the airways, cough reflexes, the phagocytic response of alveolar macrophages and secretion of immunoglobulin A. Full-blown lung inflammation is rarely necessary in healthy individuals, but mild inflammation in response to bacterial and viral infection is common (Reynolds (1987)). The inflammatory response of the lung is highly complex, involving vascular dilation, increased blood vessel permeability, plasma exudation, leukocyte infiltration, mucus secretion, shedding of epithelial cells and in some cases airway hyperresponsiveness (Barnes and Costello (1987)). The principal cell type involved in pulmonary inflammation is the polymorphonuclear neutrophil; basophilic and lymphocytic responses may also be important. The alveolus normally contains a relatively small number of polymorphonuclear neutrophils, but during inflammation chemotactic factors are released, leading to a rapid influx of inflammatory cells into the alveolus. During entry of polymorphonuclear neutrophils, the endothelium becomes damaged by proteases released by the invading cells and this, together with an increase in vascular permeability , results in accumulation of fluid containing macromolecular and cellular mediators of inflammation. After an appropriate time, the inflammatory reaction is terminated, the debris is cleared by phagocytic cells, and healing proceeds. However, if the inflammation has been severe, the normal architecture may never be restored, and in this case residual impairment and fibrosis remain (Reynolds (1987)).

Many common lung diseases include an inflammatory component.
(a) Infectious diseases: Since the development of antibiotics and vaccination, primary bacterial infections of the lung (bacterial pneumonia and pulmonary tuberculosis) have become rare, but secondary infections, causing the symptoms of the common cold and influenza, are still very common. Most cases of bacterial and viral pneumonia occur in patients whose resistance to infection is compromised for some reason, such as youth or old age, stroke or cancer. Bacterial pneumonia is an inflammatory disorder of the lung characterised by the presence of inflammatory exudate in the alveolar spaces of the lung, and can be caused by a number of organisms including Streptococcus pneumoniae, Staphylococcus aureus , Streptococcus pyogenes, Klebsiella and Haemophilus influenzae. Viral pneumonia involves proliferation of the bronchial, bronchiolar and alveolar epithelium leading ultimately to necrosis of the lung tissue and may be caused by severe infection with influenza virus or cytomegalovirus . Like pneumonia, pulmonary tuberculosis is now a rare disease and indeed has been almost eradicated from Britain. It is caused by the organism Mycobacterium tuberculosis, and

most cases are seen in patients whose immune system is suppressed, for example chronic alcoholics and those undergoing corticosteroid treatment (Anderson (1980)).
(b) Asthma: Asthma has diverse causes, both immunological (eg. allergic asthma, aspirin induced asthma, occupational asthma) and non-immunological (eg. stress-induced, exercise-induced asthma). The symptoms of asthma consist of spasmodic bronchconstriction and hyperreactivity of the conducting airways of the lung (Reynolds (1987)). The characteristic inflammatory cells involved in asthma are eosinophils; polymorphonuclear neutrophils are also involved. Macrophage and mast cell-derived factors are also important; histamine, the first mediator implicated, causes bronchconstriction, stimulates irritant receptors, opens endothelial tight junctions and is chemotactic for eosinophils whilst cyclooxygenase products are particularly significant because they bind to muscle cell receptors causing bronchospasm (Barnes and Costello (1987)).
(c) Pneumoconiosis and industrial lung disease: The major causes of industrial lung disease are dusts entering the atmosphere as a result of spinning, drilling and burning. Various factors determine the harmfulness of these dusts: these include particle size, chemical composition, concentration in the atmosphere, duration of exposure and the presence of pre-existing lung disease. Diseases such as byssinosis, caused by cotton, hemp and flax dust have become less common in this country since the demise of the English cotton industry, but are still important in countries which spin large quantities of natural fibres. Mineral dusts cause anthracosis, present to some degree in almost all adults in modern society, and coal dust leads to pneumoconiosis, an occupational hazard of coal mining. Other inorganic dusts, including iron oxide, cadmium, aluminium and tin dusts are important causes of pulmonary damage in workers who are regularly exposed in the workplace (Anderson (1980)).

Pulmonary inflammation caused by smoking is also common. Cigarette smoke contains toxic gases (eg acrolein) and inorganic particles (eg. kaolinite) which damage the delicate tissue of the lung. In an attempt to remove such substances from the lung, macrophages ingest the particles and then die, exposing small areas of the pulmonary endothelium to high concentrations of toxic and carcinogenic material and releasing damaging proteases and inflammatory mediators. Chemotactic factors released from macrophages during smoking cause accumulation of polymorphonuclear neutrophils in the lower airspaces of the lung (Brody and Davis (1982)). Complement peptide C5a and components of cigarette smoke such as nicotine are also chemotactic for neutrophils (Holt (1987), Niewoehner (1988)). Once inflammation has been

initiated by cigarette smoke, the products of proteolytic degradation of lung material maintain and exacerbate the inflammatory response (Niewoehner (1988)). Chronic inflammation induced by cigarette smoke causes hyperplasia and hypertrophy of pulmonary goblet cells and mucus glands; the resulting damage to the delicate cilia of the airways makes the lung more prone to viral and bacterial infections (Anderson (1980)). In later stages, chronic obstructive pulmonary disease can develop: cigarette smoking is the major cause of this disease, which is one of the most important causes of mortality and morbidity in modern Western society. The major form of chronic obstructive pulmonary disease is emphysema, in which the normal structural framework of the lung is destroyed as a result of the action of proteolytic enzymes such as elastase released from alveolar macrophages and polymorphonuclear neutrophils and from infective bacteria in the lung (Niewoehner (1988)). The alveolar macrophages of smokers suffering from chronic obstructive pulmonary disease release significantly more elastase than those from non-smokers or healthy smokers (McLeod et al (1985)). Proteolytic damage to components of the extracellular matrix, especially elastin and collagen, reduces the elasticity and structural stability of the lung and hence the efficiency of expansion and contraction, inhibiting respiration (Goldstein (1983)).

Although direct links between inflammation and cancer of the lung have not been established, many of the factors listed above as causing lung inflammation are also important factors in lung carcinogenesis. The most common primary malignant tumour of the lung is bronchial carcinoma, and the major causative factor for this type of tumour is smoking (Doll and Peto (1981)). The most common form of bronchial carcinoma is squamous cell carcinoma, which arises from the bronchial epithelium and forms a dense whitish mass, often with a flaky surface. Oat cell carcinoma is also common, and consists of short, darkly staining spindle cells in masses or anastomosing trabeculae. Adenocarcinoma, consisting of cuboidal or columnar epithelial cells, is less frequent; undifferentiated carcinomas, though rare, also occur (Chabner (1983), Monfardini et al (1987)). There are many ways in which the inflammation caused by pollutant dusts and gases may contribute to carcinogenesis: carcinogens may be carried into the lung on the surface of dust particles and, following damage to the pulmonary epithelium, may gain access to the lung parenchyma. Ingestion of carcinogen-carrying particles by alveolar macrophages can lead to concentration of the carcinogen within the macrophage, and when the cell dies a concentrated carcinogen solution is deposited on adjacent cells. During inflammation, a burst of cell growth occurs; the rapidly

growing cells may be more susceptible to loss of replication control as a result of mutation, and stimulation of the cells by paracrine factors produced by local inflammatory cells will produce an environment favorable to continued cell division. The importance of the lung in xenobiotic metabolism means that any adverse effect on pulmonary P-450s may have far-reaching effects on response to carcinogens such as B(a)P. The effects of inflammation on hepatic drug metabolism are already known to be significant; since the lung is susceptible to many inflammatory diseases one expects that pulmonary P-450 expression will also be affected by inflammation.

1.5.3. P-450 dependent xenobiotic metabolism in the colon.

The earliest studies on P-450 expression in the colon were those of Wattenberg in the early 1960s, which showed that AHH activity could be measured in colon mucosal microsomes from several species (Wattenberg (1962)). Colonic AHH activity was found to be somewhat lower than the hepatic level, and within the colon itself, there was a gradient of activity from higher levels in the proximal portion to lower ones in the distal portion. Immunohistochemical studies confirmed the presence of P-450s in the colon, and showed that they are concentrated in the columnar cells on the surface of the colonic mucosa (Wattenberg (1972)). Subsequent studies on P-450 expression indicated that rat colon mucosal microsomes were able to metabolise a variety of P-450 substrates including benzphetamine, ethylmorphine, p-nitroanisole and p-nitrophenol, and to activate the carcinogens B(a)P and 2-aminoanthracene (Fang and Strobel (1978), Strobel et al (1980)). Explant cultures of both normal and tumorous human colon were also shown to metabolise the carcinogens B(a)P and 1-naphthol (Cohen et al (1983)). Resolution and reconstitution of the colonic drug metabolising system of the female Sprague-Dawley rat confirmed that the system was indeed P-450-dependent and inducible. Each component was interchangeable with the equivalent hepatic component (Oshinsky and Strobel (1987a)). Western blot and radial immunodiffusion analyses demonstrated the presence of isozymes PB_1, PB_{3a}, MC_{1a} and MC_{1b} in rat colon mucosal microsomes following appropriate induction (Oshinsky and Strobel (1987b)). Isolation of P-450s from rabbit colon also indicated the presence of a w-hydroxylase enzyme possibly related to the hepatic lauric acid hydroxylase, P-452 (Kaku et al (1985). It is thought that almost all the P-450-dependent activity of the gastrointestinal tract, including the colon, is the result of induction by endogenous compounds; if rats are starved or fed a fat-free diet almost all intestinal AHH activity disappears. The fact that AHH activity is not restored

by feeding purified dietary components suggests that contaminants in the normal diet are responsible for P-450 induction (Wattenberg (1972)). In spite of the efforts of the groups mentioned above, considerably less is known about the regulation of P-450 expression in the colon than in the liver and lung. It is important that more work be performed, since P-450-dependent activation of dietary carcinogens is implicated in the development of colon cancer.

1.5.4. Inflammation and cancer of the colon.

Cancer of the colon is a "disease of Western Civilisation", in that its incidence correlates with affluence and the so-called "Western" lifestyle. Cancers of the colon and breast are the most prevalent malignancies among non-smokers in Western society (Willett (1989)). Migration studies have shown that the incidence of colon cancer in migrant populations is that of the country of residence rather than the country of origin, indicating the involvement of environmental facters in the aetiology of the disease. However, genetic predisposition is also important (Hill (1986)). Colon cancer is unusual in that the precursor stages of the disease have been clearly defined. Colonic polyps are important predisposing factors in the development of colon cancer: the results of glucose-6-phosphatase analysis suggest that polyps are multiclonal lesions within which monoclonal tumours may be initiated (Hsu et al (1983)). Sufferers from the rare genetic disease Familial Adenomatous Polyposis, in which large numbers of such polyps develop from an early age, have a very strong predisposition towards colon cancer which appears to be related to deletions in a crucial gene on chromosome 5 (Bodmer et al (1986), Solomon et al (1986)). Polyps may progress to form adenoma of the colon, a dysplasic area of the large bowel which is the precancerous lesion in the development of colon cancer. These lesions do not become malignant until the dysplasia has crossed the muscularis mucosae; relatively few adenomas progress to malignancy. The growth of adenomas may be stimulated by steroid hormones and bile acids from the colonic lumen. Progression to malignancy appears to be a function of increased severity of epithelial dysplasia (Hill (1986)). There are strong links between inflammation and carcinogenesis in the colon: inflammatory bowel diseases such as ulcerative colitis and Crohn's disease greatly increase the risk of developing colon cancer, although these diseases, being rare, account for a relatively small proportion of colon cancers. Various components of the diet have been implicated in the development of colon cancer: dietary levels of meat fat and protein appear to be particularly important. Cholesterol intake may also be involved: a high-fat, low-fibre diet appears to predispose towards colon cancer, and

alcohol is another risk factor. A possible mechanism for the effect of dietary fat is increased excretion of bile acids, which may be metabolised by the resident bacteria of the gastrointestinal tract to produce carcinogens and tumour promoters. Amino acids which are found in the gut contents may also be metabolised to mutagenic derivatives by the intestinal flora. The resulting carcinogens may be susceptible to further activation by intestinal P-450s. Dietary fibre is an important protective factor against colon cancer, although the suggestion that increased fibre intake per se is the protective agent is an oversimplification. The decreased risk of colon cancer appears to relate to fruit and vegetable intake, and may be due to vitamins, β-carotene, indoles or other components in these foods. There is no evidence that increased intake of cereal fibre protects the colon from carcinogenesis (Hill (1986), Willett (1989)). In view of the involvement of both dietary carcinogens, which may be activated or detoxified by P-450s, and inflammation in the aetiology of colon cancer it is clear that a more detailed understanding of the regulation of P-450s in the colon both by inducing agents and during inflammation is required.

1.6. Aims of this thesis.

The aim of this project was to study the regulation of P-450 protein expression and enzyme activity in a range of human tumour-derived cell lines, with a view to choosing one as a model in which to investigate P-450 regulation by inflammatory mediators. Human tumour-derived cell lines were chosen for this work because they represent a system in which the response of a single cell type to P-450 inducers and inflammatory mediators can be examined under controlled conditions.

Inflammation causes suppression of constitutive P-450-dependent drug metabolism in the liver; this appears to be mediated by interferons and other lymphokines, although it is not clear exactly which mediators are involved. This aspect of P-450 regulation merits further research since it is important in certain circumstances: in humans, susceptibility to both the adverse and beneficial effects of drugs is altered during infection; this factor should be taken into account in attempting to predict therapeutic responses, particularly to drugs which have a low therapeutic index. Susceptibility to environmental carcinogens may also be affected; this could be important in human populations exposed to irritants as well as carcinogens, either in the workplace or as a result of tobacco smoking. Sub-clinical infections in experimental animals might significantly affect the results obtained in toxicity and oncogenicity testing: Renton

(1983) reports that he has obtained, from reputable suppliers, animals whose P-450 levels were unusually low and were refractory to further suppression during inflammation, possibly due to sub-clinical infection.

The major problem with early studies implicating interferon in P-450 modulation is that relatively non-specific agents were used. Compounds such as endotoxin and tilorone, as well as inducing interferon, exert a number of other effects and it is difficult, if not impossible, to demonstrate that interferon is the actual mediator of their effects on P-450s. Early attempts to use "purified" interferons and other lymphokines were not much more successful because the preparations used were very crude; with the availability of highly purified recombinant DNA-derived lymphokines it is now possible to attempt the identification of specific mediators involved in the suppression of P-450s, and a number of reports now exist of attempts to do so. One of the aims of the present project was to attempt a survey of the effects of several recombinant DNA-derived lymphokines on P-450 expression.

Another problem with these studies is that the criteria used to assess effects on the P-450 system were extremely general; activities such as 7-EC O-deethylation are mediated by several P-450 isozymes, total P-450 encompasses all P-450 isozymes present and drug clearance is an even more vague parameter, depending not only on a number of P-450s but also on rates of blood flow, binding of drugs to serum albumin and activities of conjugating enzymes. If the effects of inflammation on specific isozymes are to be understood as fully as those of inducing agents, it is necessary to use specific methods to examine different members of the P-450 superfamily. Use of more clearly defined enzyme activities (dealkylation of resorufin ethers) and immunological techniques (Western blotting) to elucidate the effects of lymphokines on specific P-450 isozymes will form part of the present work; further techniques such as Northern blot analysis using specific P-450 cDNA probes would represent an even more specific approach to the problem.

As indicated above, previous studies on the effects of inflammation have concentrated on constitutive P-450-dependent activities; few studies have examined the effects of concomitant treatment with a P-450 inducer and an inflammatory agent. Those studies

which have done so suggested that endotoxin did suppress inducible P-450-dependent activities but inducing agents, including PB, 3-MC, SKF 525A and Aroclor 1254 offer some protection against interferon-mediated P-450 suppression . In each of these studies only one, or at most two, parameters of P-450 expression were measured and the specific isozymes involved were not identified (Reiners et al (1984), Tanaka et al (1985), Matsunaga et al (1986a,b)). Since most P-450 inducers affect many isozymes, these studies are insufficient for an understanding of their interaction with interferon in P-450 regulation. Previous work in this laboratory suggested that low doses of endotoxin or ifn α could in fact potentiate P-450 induction by PB and 3-MC (C.R.Wolf, personal communication). If it is the case that these agents potentiate P-450 induction, susceptibility to carcinogens could be dramatically increased during inflammation. In this project the effects of treatment of mice with PB or 3-MC together with an inflammatory agent (E. coli endotoxin) or an interferon (ifn α) will be considered.

In the intact animal complex cellular, hormonal, paracrine and autocrine mechanisms are involved in inflammation. It will ultimately be necessary to attempt a complete analysis in vivo, but a cell culture model represents a simplified system in which to survey mediators for their capacity to affect P-450s. To date, studies of the mechanism of the effects of interferon on P-450 have been hampered by the lack of a suitable cell model. The levels of P-450 expressed by replicating cells in culture are too low to be detected by the methods used to study P-450s in liver microsomes, and in the past non-replicating hepatocytes were the only model available for experiments on P-450 expression in vitro. These cells lose almost all their P-450s within 24 hr in culture, raising questions about their usefulness for studies on P-450 suppression. Experiments using primary hepatocytes have been reported: an early study reported that a crude interferon preparation actually induced P-450 in foetal hepatocytes after 24 hr in culture (Renton et al (1978)). In this context it is interesting that in the foetus and neonate the response of P-450s to interferon is markedly different from that in the adult (Robbins and Mannering (1984 a,b)). It appears that the changes in hepatic metabolism which take place at parturition include a dramatic alteration in the response of the P-450 system to interferon. The alternatives to primary hepatocytes for the in vitro study of P-450 regulation are continuously cultured cell lines; use of

a human cell culture model means that tumours of various origins may be studied. Previous work has concentrated on the liver because this is quantitatively the major organ involved in drug metabolism, but the lung and colon also have the capacity to perform P-450-dependent drug metabolism and are susceptible to inflammation. Cell lines derived from human liver, lung and colon tumours were chosen for detailed study in this project; the aim was to identify a cell line in which to carry out a survey of the effects of different lymphokines on extrahepatic P-450 expression.

In this project, therefore, the regulation of P-450 expression in five human tumour-derived cell lines, one of liver origin (HepG2), two of lung origin (NCI H322, NCI H358) and two of colon origin (HT29, LS174T) was examined. In the experiments discussed in Chapter 3, the response of the cell lines to P-450 inducers was assessed using immunological and enzymatic methods and the results were used to choose a cell line for further study. Induction of P-450 was confirmed by Northern blot analysis and the conditions for induction were optimised. In Chapter 4 experiments to optimise the "MTT assay" for two cell lines are described. This assay was used to estimate the toxicity of P-450 inducers and to examine the effects of P-450 induction on the in vitro toxicity of B(a)P and cyclophosphamide, which are susceptible to activation by different P-450 isozymes. Chapter 5 describes experiments designed to assess the effects of a variety of inflammation inducers and mediators on P-450 expression. The first model used was the male CBA mouse liver, in which studies using E.coli endotoxin and interferon α were confirmed and extended to show that both these factors can have marked effects both on P-450 protein expression and P-450-dependent enzyme activities. The other model was the human lung cell line NCI H322, whose response to various recombinant cytokines was assessed using the MC_{1b} substrate 7-ethoxyresorufin. It is hoped that the experiments described in this thesis will establish the usefulness of human tumour-derived cell lines of extrahepatic origin in studying the capacity of exogenous and endogenous factors to regulate P-450 expression.

Chapter 2.
Materials and Methods.

2.1. Sources of materials.

All chemicals were obtained from BDH Limited, Burnfield Avenue, Thornliebank, Glasgow, G46 7TP wherever possible. Cell culture materials were obtained from Gibco Limited, P.O. Box 35, 3, Washington Road, Paisley, PA3 4EP unless otherwise stated. A list of chemicals and suppliers used is given in Appendix 1.

2.2. Animal Experiments.

2.2.1. Induction experiments using mice.

Animal experiments were performed at the Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh or at the Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX. Male CBA mice (25g) were fed on standard laboratory chow and allowed to acclimatise for 7 days in a controlled environment with a 12 hour light/dark cycle, then treated intraperitoneally with sodium phenobarbitone (80mg/kg body weight in 0.9% NaCl) or 3-methylcholanthrene (20mg/kg body weight in corn oil) for three days. Endotoxin from E. coli or recombinant α interferon was administered as described in Chapter 5. The animals were starved overnight on day three of the experiment and sacrificed by cervical dislocation on day four. The livers were removed, washed with 10mM phosphate buffer pH 7.4 containing 1.15% KCl, immediately placed on ice, then stored at -40^{o}C until required.

2.2.2. Preparation of microsomal fractions from mouse liver.

Microsomal fractions were prepared according to the method of Adams et al (1985). Liver samples were thawed rapidly, scissor-minced and homogenised using a

Silverson Laboratory Mixer Emulsifier in 3 volumes (i.e. 3ml per gram wet tissue) of 10mM phosphate buffer pH 7.4 containing 1.15% (w/v) KCl and 0.1mM EDTA. All operations were carried out at 4°C. Homogenates were centrifuged at 11,000*g* (11,000 rpm) for 20 min in a Du Pont Sorvall RC-5B Refrigerated Superspeed Centrifuge (SS 34 rotor) to remove cell debris, and the supernatants centrifuged at 230,000*g* (45,000rpm) for 1h in a Du Pont Sorvall OTD65B Ultracentrifuge (TFT 45.6 rotor). The supernatant (cytosolic fraction) was removed and the microsomal pellet washed with the same buffer and recentrifuged for 40 min at 230,000*g* (40,000rpm). The microsomal samples were resuspended in ice cold 0.25mM sucrose/10mM potassium phosphate pH 7.4 to a protein concentration of 10 - 20mg/ml using a Teflon-glass homogeniser, aliquotted in volumes of ~1ml and stored at -40°C until required. In some cases samples were stored for up to 18 months before use, but it was found that if they were not subjected to more that one freeze-thaw cycle the levels of P-450-dependent activities were maintained, in agreement with the data of Danner-Rabovsky and Groseclose (1982) which indicated that P-450 activities in frozen tissues or microsomes stored at -80°C were stable for several months.

2.3. Experiments using human tissue samples.

2.3.1. Sources of human material.

Samples of human lung parenchyma and lung tumours were obtained from patients undergoing surgery at the City Hospital, 51 Greenbank Road, Edinburgh. Tissue was obtained at thoracotomy in 10 previously untreated patients, biopsies of tumour and normal lung (from a site distal to the tumour) being taken and placed on ice. Samples were snap frozen in liquid nitrogen as soon as possible then stored below -40°C until required. Samples of human colon mucosa and tumours were obtained from Professor Adrian Harris, University Department of Clinical Oncology, Newcastle General Hospital, Westgate Road, Newcastle-upon-Tyne. These samples were removed from colon cancer patients during tumour resection; "normal"mucosa was identified histopathologically*. Again, samples were snap-frozen in liquid nitrogen as soon as possible after resection then maintained below -40°C until required. Subcellular fractions were prepared as described below, the microsomal fraction being retained for studies on P-450 expression and the cytosolic fraction used for studies on glutathione-dependent enzymes (Carmichael et al (1988)). The available clinical data concerning these samples is summarised in Table 2.1.

* It should, however, be noted that the non-tumour material from a tumour-bearing lung or colon cannot be considered to be biologically normal, since factors released by the tumour may affect the behaviour of other cells within the organ. Ideally, it would have been preferable to include lung and colon microsomes from healthy subjects as well as tumour and non-tumour material from cancer patients in this study, but for ethical reasons such samples are very rarely available.

Table 2.1.

Clinical data on human tumour samples.

(a) Human Lung Samples.

Code No.	Initials	Sex	Date resected	Tumour Type*	Differentiation
1	KP	F	22.03.83	Squamous	Poor
2	KH	M	08.03.83	Adenocarcinoma	Poor
3	JM	M	24.02.83	Large Cell	N K
4	LH	M	06.04.83	Small Cell	N K
5	AM	M	14.03.83	Squamous	Poor
6	RM	M	15.03.83	Squamous	Poor
7	TB	M	09.03.83	Adenosquamous	N K
8	MS	F	25.03.83	Squamous	Poor
9	AL	M	24.05.83	Squamous	Moderate
10	FB	M	01.03.83	Large Cell	N K

(b) Human Colon Samples.

Code No.	Patient	Sex	Age	Duke's Status+	Location	Differentiation
1	87/2917	M	58	B	Rectum	Moderate
2	87/2936	M	51	A	Rectum	Moderate
3	87/5430	M	73	C	Sigmoid colon	Moderate
4	87/5576	M	64	A	Rectum	Moderate
5	87/5909	M	55	N K	N K	N K
6	87/7950	F	70	N K	N K	N K
7	87/9054	M	66	N K	N K	N K
8	87/9387	M	69	N K	N K	N K
9	87/9629	F	67	N K	N K	N K

* Human lung tumours are classified histologically into a range of sub-types exhibiting variation in patterns of growth, response to treatment and prognosis (Monfardini et al (1987))
+The most commonly used system for classifying colon tumours and assessing their prognosis is that of Duke, summarised in Monfardini et al (1987).
N K - not known.

2.3.2. Preparation of subcellular fractions from human extrahepatic material.

Preparation of microsomal and cytosolic fractions from human samples was carried out essentially as described above, with the following modifications:

(a) In order to protect the operator from pathogens carried by aerosols formed during homogenisation of human samples, the first stages of preparation were carried out in a MDH Class II Laminar Flow Hood and a surgical mask was worn. Microtouch latex surgical gloves were worn throughout the preparative procedure.
(b) Since lung and colon contain large amounts of connective tissue, it was not possible to homogenise the samples completely without maintaining them for excessive periods at room temperature. The samples were therefore homogenised briefly; remaining fibrous material precipitated with the cell debris during the first centrifugation step.
(c) The microsomal content of human lung and colon is low compared to that of liver. The final protein concentration of stored microsomal samples was 2 - 10mg/ml.

2.4. Cell Culture.

2.4.1. Cell lines.

Five human cell lines were studied in this project. They were chosen because they had previously been shown to perform P-450-dependent drug metabolism or were derived from the lung or colon, tissues in which inflammation is implicated in carcinogenesis. The cell line HepG2 was derived at the Wistar Institute from a childhood hepatoblastoma (Knowles et al (1980)) and has been shown to retain a number of features found in differentiated hepatocytes as well as detectable levels of P-450- dependent drug and carcinogen metabolising activities. Two non-small cell lung carcinoma lines derived in the laboratories of Dr A.F. Gazdar and Dr J.D. Minna at the National Cancer Institute, Bethesda, Maryland were chosen because detailed studies of their ultrastructure (Schuller et al (1985)) have enabled their probable cell type of origin to be identified. NCI H322 is thought to be derived from the Clara cell (the cell type thought to express the majority of lung P-450-dependent activity) and NCI H358 from the Type II pneumocyte. Two colon lines were also available for study. The first, HT29, was derived from the primary adenocarcinoma of a 44 year old female Caucasian patient by Dr J.Fogh at the Memorial Sloan-Kettering Cancer Center (Fogh and Trempe (1975)). The second line, LS174T, was derived at the Northwestern University Medical Center by Tom et al (1976). It arose from a moderately well differentiated colon adenocarcinoma in a 58 year old female patient. This tumour gave rise to two cell lines, LS174T and LS180, both of which have 45 chromosomes and produce carcinoembryonic antigen and mucin. The sources from which the cell lines were obtained are listed in Table 2.2.

Table 2.2.

Sources of human cell lines used in this project.

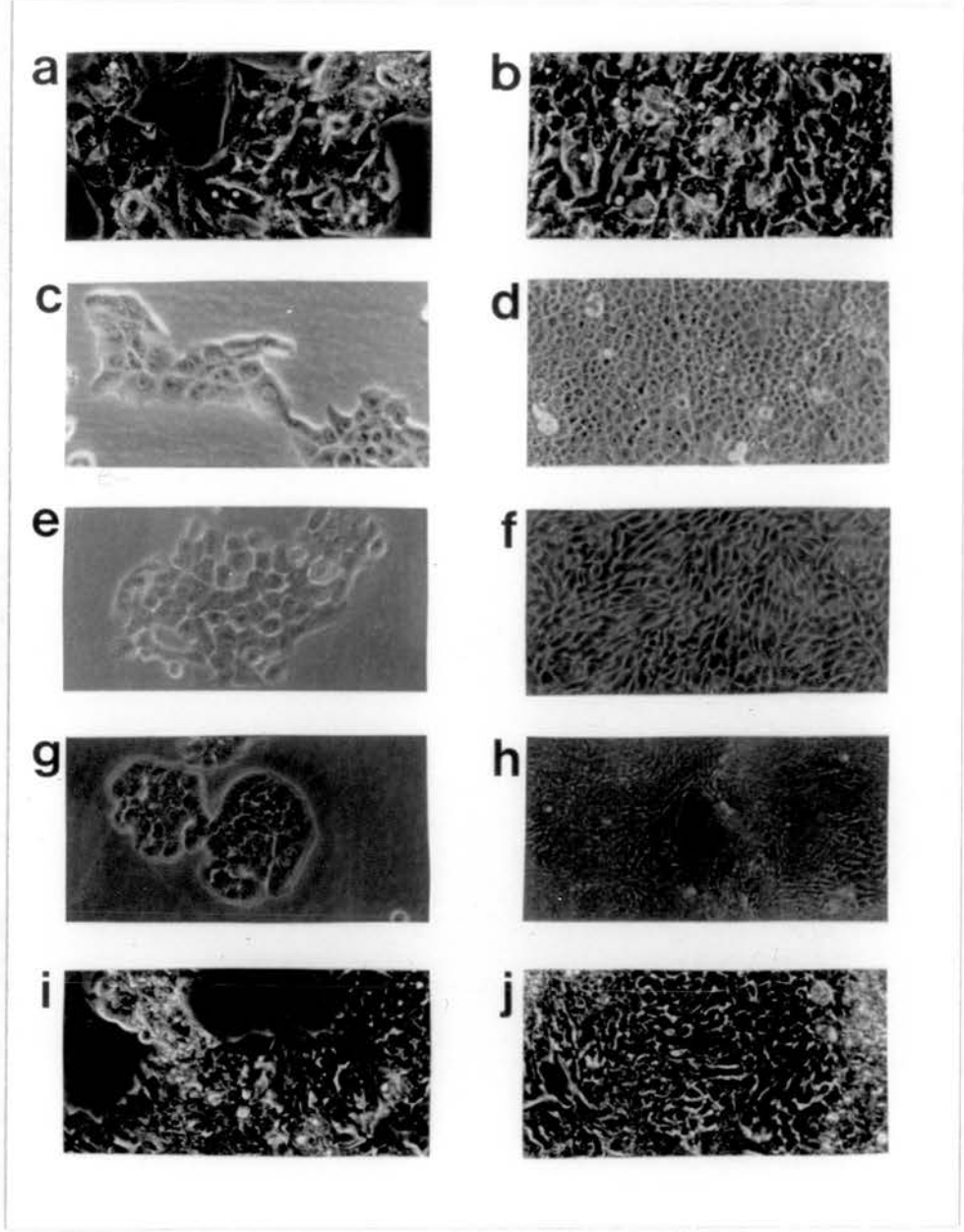
Cell line	Source of stocks
HepG2	(1) European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology and Research, Vaccine Research and Production Laboratory, Porton Down, Salisbury, Wilts, SP4 0JG. (2) Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX.
NCI H322, NCI H358	Dr. J. Carmichael, National Cancer Institute-Navy Medical Oncology Branch, National Cancer Institute and Naval Hospital, Bethesda, Maryland 20814, USA.
HT29	Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX
LS174T	(1) Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX. (2) European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology and Research, Vaccine Research and Production Laboratory, Porton Down, Salisbury, Wilts, SP4 0JG.

It was important to obtain cell lines from reputable sources, since there is evidence that many stocks of human cell lines have been contaminated with cells from other lines and even other species. In the 1960s it was shown that HeLa cells are a particularly common contaminant of human tumour-derived cell lines (Gartler (1967,1968)), but with the development of techniques for analysing marker chromosomes (Nelson-Rees et al (1974)) and enzyme polymorphisms (Montes de Oca et al (1969), O'Brien et al (1977,1980)) the authenticity of certain cell lines was confirmed (Fogh et al (1977), Wright et al (1981)). Cells obtained from such sources as the American Type Culture Collection (stocked by the Porton Down Repository) or the National Cancer Institute may be assumed to be authentic.

The morphology of the cell lines used is shown in Figure 2.1. HepG2 cells grew in

Figure 2.1.

Morphology of human tumour-derived cell lines.



Logarithmic and confluent cultures were photographed at a magnification of 100 - 150x using an Olympus C-35DA-2 automatic camera attached to an Olympus TO41 phase contrast microscope. White light (setting 12) was passed through a green filter and the exposure set at 0.1 seconds.

(a) HepG2 - Logarithmic phase
(b)HepG2 - Confluent phase
(c) NCI H322 - Logarithmic phase
(d)NCI H322 - Confluent phase
(e) NCI H358 - Logarithmic phase
(f)NCI H358 - Confluent phase
(g) HT29 - Logarithmic phase
(h)HT29 - Confluent phase
(i) LS174T - Logarithmic phase
(j)LS174T - Confluent phase

monolayer form (doubling time = 60hrs) until reaching confluency but lifted off the substratum at this stage. Both lung cell lines formed rapidly growing monolayers (doubling time of NCI H322 = 30hrs) and remained adhered but stopped growing upon reaching confluency. HT29 cells consisted of quite small cells which grew rapidly and readily became confluent, but LS174T cells were exceptionally small and grew slowly, forming tightly packed colonies which often developed necrotic centres and frequently lifted off into the medium without acheiving confluency.

2.4.2. Cell Culture Techniques.

Cell culture methods were carried out essentially as described by Freshney (1987).
(a) Cryopreservation of cells: Cell lines were stored in liquid nitrogen as follows: Confluent cells were harvested using 0.1% trypsin + 0.01% EDTA , washed with growth medium and resuspended at a cell density of 5-10 x 10^6/ml in 90% newborn calf serum with 10% DMSO. The samples (~1ml) were frozen initially at -70^{o}C for 24hr then kept in liquid nitrogen (-196^{o}C) until required. DMSO was chosen as the cryopreservative because it permeates cells rapidly and allows good maintenance of viability during long-term storage (Lovelock and Bishop (1959)). When required, cell samples were thawed rapidly at 37^{o}C, washed in 20ml of growth medium, transferred to 25cm^2 flasks and allowed to adhere overnight before refeeding.
(b) Refeeding: Cells were refed by pipetting off the spent medium and replacing it with fresh medium at 37^{o}C. Refeeding was carried out as required (usually two or three times weekly) in order to maintain stable pH and optimal cell growth. NCI H322, NCI H358 and HT29 cells were maintained on RPMI 1640 medium (Moore et al (1967)) supplemented with 10% foetal calf serum (FCS). HepG2 and LS174T cells were maintained on Minimum Essential Medium (Eagle (1959)) supplemented with 15% FCS (HepG2) or 10% FCS and non-essential amino acids (LS174T). In some experiments, Dulbecco's modification of Eagle's medium (DMEM) (Morton (1970)) or William's E medium supplemented with 10% FCS were used. Glutamine (0.2mM) penicillin (15u/ml) and streptomycin (5µg/ml) were added to all media.
(c) Subculture: Cells were subcultured upon reaching confluency by washing three times with phosphate buffered saline (PBS) then treating with 0.1% trypsin + 0.01% EDTA at 37^{o}C until the cells detached from the growth surface. Fresh medium with serum was added to inhibit the trypsin and the cells pipetted until a single-cell suspension was obtained. The cells were washed with fresh medium, centrifuging for 5 minutes at 2500rpm in a MSE Microcentaur centrifuge. They were counted

using a Neubauer haemocytometer and their viability assessed by nigrosin dye exclusion (Kaltenbach et al (1958)) if necessary, then seeded into new flasks. For most cell lines the seeding density was not critical over the range 10^4 - 10^6 cells/cm^2 although for LS174T cells it was necessary to seed at a density of close to 40,000 cells/cm^2 in order to obtain healthy cultures.

(d) Sterility: Sterility was maintained in the cell culture suite and cell culture techniques were carried out aseptically. Solutions were sterilised in a Laboratory Thermal Equipment 225 EH autoclave if possible; those which could not be autoclaved were passed through a 0.22μm filter before storage and again before use. Sterility testing was carried out by inoculating a sample of each solution into L-broth (2% Bactotryptone, 1% Bactoyeast, 2% NaCl) and incubating at 37°C for at least a week. If turbidity was observed, the solution was discarded and a fresh sterile stock made. All cells used were free from mycoplasma contamination, as demonstrated by regular mycoplasma tests carried out by Mr. W. Christie in the MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh. The tests used involved staining with Hoescht 33258 fluorescent dye (Chen (1977)) and growth on selective broth (Taylor-Robinson (1978)). It was important to use mycoplasma free cells in this work since mycoplasma infection affects the response of cultured cells to P-450-inducing agents (Bradlaw et al (1982)) and interferon (Barile (1973)), and causes other changes including chromosome aberrations and loss of tumorigenicity in nude mice (Fogh and Fogh (1965), van Diggelen et al (1977)).

2.4.3. Induction of cytochrome P-450s in cultured cells.

The concentrations of inducing agents applied to cell lines were:

Phenobarbital	1 - 5mM
Dexamethasone	10μM
Aroclor 1254	3 - 9μg/ml
1,2-Benzanthracene	3 - 9μg/ml (13 -39μM)

Phenobarbital was readily soluble in aqueous solvents so a filter sterilised stock of 100mM in PBS was used. Dimethylsulphoxide (DMSO) was chosen as the solvent for other inducing agents since it is a good solvent for hydrophobic compounds and is readily miscible in aqueous media. The inducing agents were made up as concentrated stocks in DMSO such that the final concentration of DMSO in the medium would be ~0.1%. Although DMSO does have biological effects on cultured cells, including extended viability, maintenance of differentiation and morphological changes in primary hepatocytes (Muakkassah-Kelly et al (1987), Isom et al (1985)) and

tumour-derived cell lines (Higgins et al (1983)), these effects are observed at relatively high concentrations (1 -2% v/v) after at least 48h exposure, so it was assumed that the low concentrations used in these experiments would not significantly affect the behaviour of the cells. This was borne out by the lack of any morphological or cytotoxic effect of DMSO at the concentrations used.

The choice of concentrations of cytokines to apply to cells was made on the basis of literature reports of their biological effects. The concentration of endotoxin chosen (1μg/ml) has been shown to elicit a number of biological effects including induction of tumour necrosis factor (TNF) and interleukin-1 (IL-1) mRNA synthesis and protein secretion by macrophages (Koerner et al (1987), Gifford and Lohmann-Matthes (1987)) and ifn β_2 secretion by fibroblasts (Helfgott et al (1987)). The choice of an appropriate concentration of TNF was more difficult as use of a wide variety of concentrations of this cytokine has been reported; often only the cytotoxic effects of TNF were studied. It was decided to use a concentration which was in the upper range of those previously reported, but was non-toxic to the majority of cell lines studied. Many cell lines appear to be resistant to the toxic effects of TNF at concentrations of 0.2 -1.3 μg/ml, although one human lung tumour-derived cell line (A549) does suffer about 10% growth inhibition at 20ng/ml (Fransen et al (1986), Schiller et al (1986, 1987)). A concentration of 500 ng/ml was chosen for experiments with NCI H322 cells. Most of the biological effects of IL-1 occur at concentrations of 10pg - 10ng/ml, and 10ng/ml is considered to be a very high concentration (F. DiGiovine, personal communication). On this advice, a concentration of 1ng/ml was chosen for these experiments. Dex was used at the same concentration as that used to screen cell lines for P-450 inducibility (10μM). Interferon has been shown to affect P-450 expression in primary mouse hepatocyte culture at a concentration of 1000 u/ml (Renton et al (1978)). Interferons also exert other effects at this concentration, which appears to be non-toxic to a variety of human tumour-derived cell lines (Fidler et al (1987), Boraschi et al (1987), Elias et al (1987), Black et al (1987)). Recombinant interferons were therefore used at a concentration of 1000u/ml.

Cells for induction experiments were seeded into 25cm^2 or 75cm^2 flasks at a density of approximately 1 x 10^5 cells/cm^2 and allowed to grow until just sub-confluent. The cells were then refed with fresh medium containing inducing agent (or DMSO as

control). In some experiments lymphokines were dissolved in PBS and added to the culture medium at the same time as the inducing agent. The cells were exposed to inducing agent, with or without lymphokine, for 24 hours at 37°C then harvested, washed with 20ml PBS and resuspended in ice cold 10mM potassium phosphate with 0.25mM sucrose pH 7.4 (300 - 500µl). Each sample was sonicated (3 x 1 sec) using a MSE Soniprep 150 sonicator and stored at -40°C until required.

2.5. Assessment of protein content of samples.

2.5.1. Protein Estimation.

The protein content of samples was estimated according to the method of Lowry et al (1951) using bovine serum albumin as standard. The relationship between protein concentration and OD_{600nm} of the final complex approximated to linearity over the range 0 -200µg/ml; all samples were diluted in 0.1M NaOH to be within this range. Optical densities were measured using a Shimadzu UV 160 spectrophotometer and the protein concentrations derived from a newly assayed standard curve on each occasion.

2.5.2. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Before Western Blot analysis was performed, samples were assessed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) to ensure that the protein estimations were accurate and that the samples were not degraded. By this method it was also possible to compare the relative intensities of bands in the molecular weight range of P-450s in mouse liver microsomes, but in cultured cells the level of P-450 expression was too low for these bands to be visible on a Coomassie Brilliant Blue stained gel. Samples were prepared for electrophoresis by diluting to a concentration of 3mg protein/ml with 100mM Tris-HCl pH 7.4, adding an equal volume of "boiling mix" (0.05M Tris-HCl pH 6.8 containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol and bromophenol blue dye to give a deep blue colour) and heating to 100°C for 2 - 10 minutes. The amount of protein loaded for Coomassie Brilliant Blue staining of cell sonicates or microsomes was usually 25µg. Molecular weight marker mixtures containing alpha-lactalbumin (Mr=14,200), soybean trypsin inhibitor (Mr=20,100), trypsinogen (Mr=24,000), bovine erythrocyte carbonic anhydrase (Mr=29,000) rabbit muscle glyceraldehyde-3- phosphate dehydrogenase (Mr=36,000), chicken egg albumin (Mr=45,000) and bovine serum albumin (Mr=66,000) were used at

concentrations such that when 10µl was loaded, each band was clearly visible. SDS-PAGE gels (0.15 cm thick) were cast using a Biorad Protean II electrophoresis apparatus. The separating gel consisted of 9% (w/v) polyacrylamide (8.7% acrylamide with 0.3% N,N^{l}-methylene-bis-acrylamide) in 0.375M Tris-HCl pH 8.8 with 0.15% SDS. Ammonium persulphate (APS) (0.05%) and N,N,N^{l},N^{l}-tetra-methyl ethylenediamine (TEMED) (0.05%) were the setting agents used. The gel was poured to a height of 12cm and immediately overlaid with distilled water before being allowed to set for approximately 30mins. The overlay was then poured off, a well-forming comb (15 to 25 tracks) inserted and stacking gel (4.5% (w/v) acrylamide in 0.125M Tris-HCl pH 6.8 with 0.125% SDS, 0.05% APS and 0.05% TEMED) poured to the top of the plates. If the gels were to be stored overnight they were covered with a double layer of clingfilm and placed at 4^{o}C at this stage. When the gels were required, the combs were carefully removed and the assembled gel attached to the central cooling reservoir of the Protean II apparatus. The top buffer reservoir was filled with electrode buffer (0.05M Tris-HCl pH 8.3 with 0.05M glycine and 0.1% SDS) and the samples loaded using a Hamilton syringe. The gel kit was assembled according to the manufacturer's instructions and the gels run at 20 - 40 mA with cold water cooling until the dye front was approximately 1cm from the base of the gel (~6h at 20mA). The gels were removed from the glass plates, fixed in 45.5% methanol with 9% acetic acid for at least 30min, stained with 0.25% Coomassie Brilliant Blue in 45.5% methanol with 9% acetic acid for 1hour, and destained in 10% methanol with 7% acetic acid until bands were clearly visible.

2.6. Immunochemical Methods.

2.6.1. Characterisation of anti-P-450 antisera.

Antisera to purified P-450 isozymes (Wolf and Oesch (1983), Wolf et al (1984, 1986)) were supplied by Dr C.R.Wolf. The antisera were characterised by Ouchterlony double immunodiffusion and Western blot analysis.

(a) Ouchterlony double immunodiffusion analysis: Ouchterlony gels containing 0.9% Type II Agarose, 0.08M NaCl, 1.0M glycine and 0.015M NaN_3 were poured in Miles Laboratory immunodiffusion plates and 10µl of sample loaded in each well. Purified rat liver P-450s were used at 0.25mg/ml and antisera at a dilution of 1/2 in O.1M Tris-HCl pH 7.4. Plates were incubated at room temperature in a humid atmosphere until white precipitin bands appeared (~5 days), then washed for 24h in 0.9% NaCl

with 0.05% Tween 20 and 0.02% NaN_3, dried onto the hydrophilic side of Gelbond film and stained with Coomassie Brilliant Blue (SDS-PAGE stain diluted 1/2 with H_20). The precipitin pattern was interpreted as described by Roitt (1988).

(b) Western blot analysis: The cross-reactivities of the antisera were assessed by Western blot analysis as described below. The gels were overloaded with antigen (10μg) in order to detect slight cross-reactivity; each antibody was used at a dilution of 1/500. The results are summarised in Table 2.3. Antisera raised against the inducible isozymes PB_{2c}, PB_{3a} and MC_{1b} were the most specific, showing little cross-reactivity with other isozymes. The PB_1 antiserum did cross-react slightly with other P-450s, particularly those of PB-inducible families.

Table 2.3.

Characterisation of antisera against cytochrome P-450 isozymes.

(a) Ouchterlony double diffusion analysis.

	Antiserum: Anti-PB_1	Anti-PB_{2c}	Anti-PB_{3a}	Anti-MC_{1b}
Antigen:				
PB_1	++++	+	++	++
PB_{2c}	+++	+++	+	++
PB_{3a}	+	+	+++++	+
MC_{1a}	++	+	+	++++
MC_{1b}	-	+	+	+++++

(b) Western Blot Analysis.

	Antiserum: Anti-PB_1	Anti-PB_{2c}	Anti-PB_{3a}	Anti-MC_{1b}
Antigen:				
PB_1	+++++	-	-	-
PB_{2c}	++	++	+	-
PB_{3a}	+++	+	+++++	++
MC_{1a}	+	-	+	++++
MC_{1b}	+	-	-	+++++

Double diffusion and Western blot analyses were carried out as described and the intensity of the precipitin bands on Ouchterlony plates or the bands on Western blots assessed by eye. A + sign indicates that cross-reaction occurred; the number of + signs gives an estimate of intensity. A - sign indicates lack of cross-reactivity.

2.6.2. Dot blot analysis.

Two immunochemical methods, dot and Western blot analysis, were used to analyse P-450 expression in microsomes and cell lines. Dot blot analysis was the more preliminary method; it was used to screen cell lines for P-450 inducibility and to ensure that the antigen-antibody reaction taking place on nitrocellulose was linear with respect to antigen loading. Samples were dotted onto nitrocellulose sheets in a volume of 1μl using a Gilson pipette. They were allowed to dry completely and then blocked and labelled in the same way as Western blots on microsomal samples. To show that the relation between amount of antigen loaded and labelling intensity was linear, a dot blot was carried out loading 0.25 - 250ng of four purified P-450s (PB_1, PB_{2c}, PB_{3a} and MC_{1b}) and labelling with the corresponding antisera. The blot was labelled using ^{125}I labelled protein A (see below), each square of the grid cut out and the extent of labelling measured by gamma-counting. The results showed that in each case the relationship between amount of antigen loaded and number of counts bound approximated to linearity (Figure 2.2). This experiment also showed that the limit of detection of the method was approimately 1ng of purified antigen. A second experiment was carried out to assess the variability of labelling between dots and to show that the intensity of the dots on an autoradiograph was proportional to the number of counts bound (Figure 2.3). This showed that the variability between dots was small except when a large amount of antigen (250ng) was loaded.

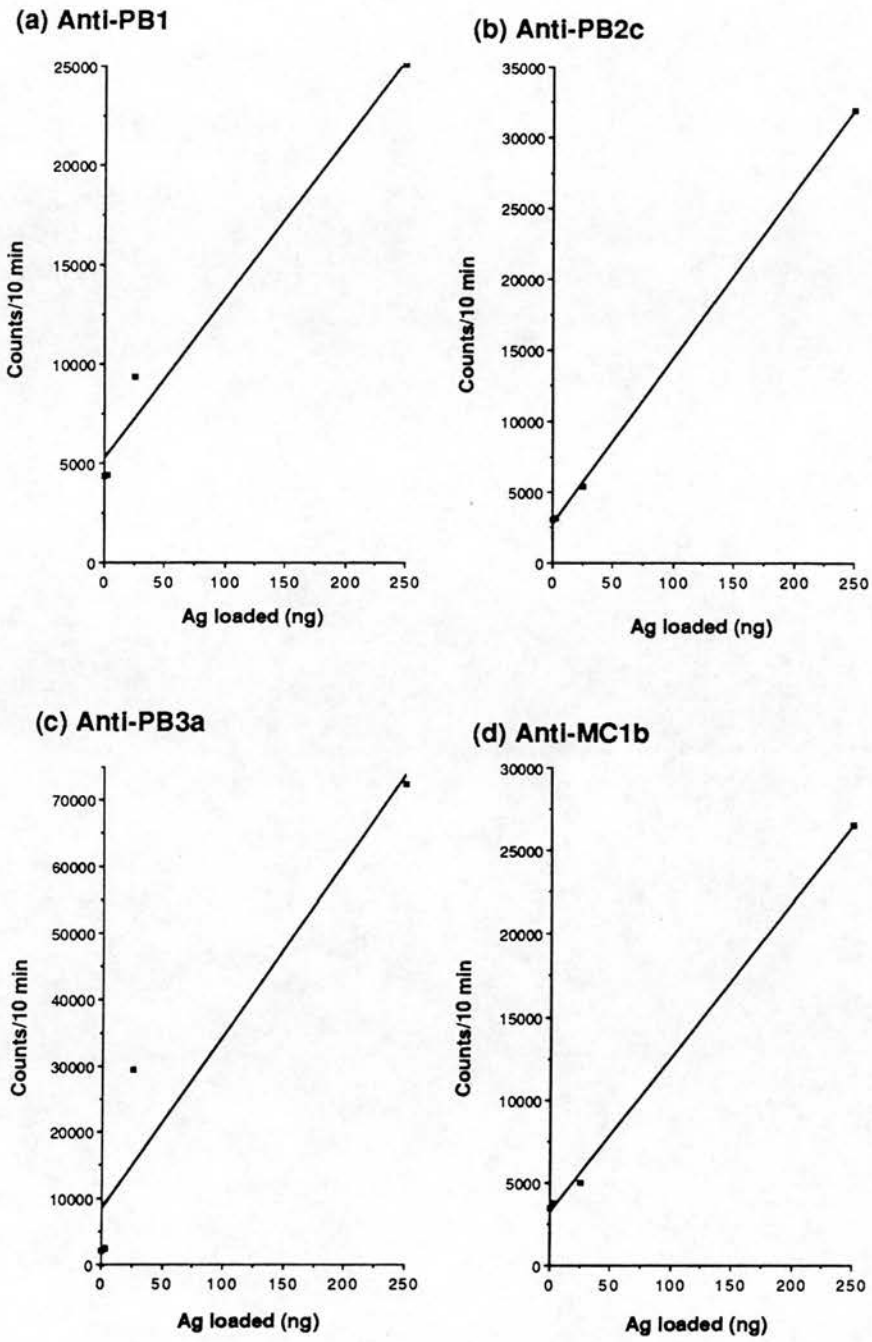
2.6.3. Western Blot analysis.

Western blot analysis was performed by a modification of the method of Towbin et al (1979). For analysis of rodent liver microsomal samples, dual labelling with horse radish peroxidase-conjugated goat antiserum against rabbit immunoglobulin G (IgG) followed by ^{125}I conjugated protein A was used. The method was further modified in order to detect P-450s in cell lines and human extrahepatic microsomes.

(a) Detection of P-450s in rodent liver microsomes: SDS-PAGE was performed as described in Section 2.5.2, loading 7.5μg of microsomal protein per track. Following electrophoresis unwanted parts of the gel were removed, orientation markings were made, and it was placed adjacent to a 0.45μm nitrocellulose filter in a Biorad transblotting cassette with Whatman 3mm paper and "Scotchbrite" pads to hold it in place. The cassette was placed in the Biorad transblotting apparatus with the nitrocellulose nearest to the anode and transblotted overnight at 250mA. After transblotting, the nitrocellulose was cut to the exact size of the gel and the necessary

Figure 2.2.

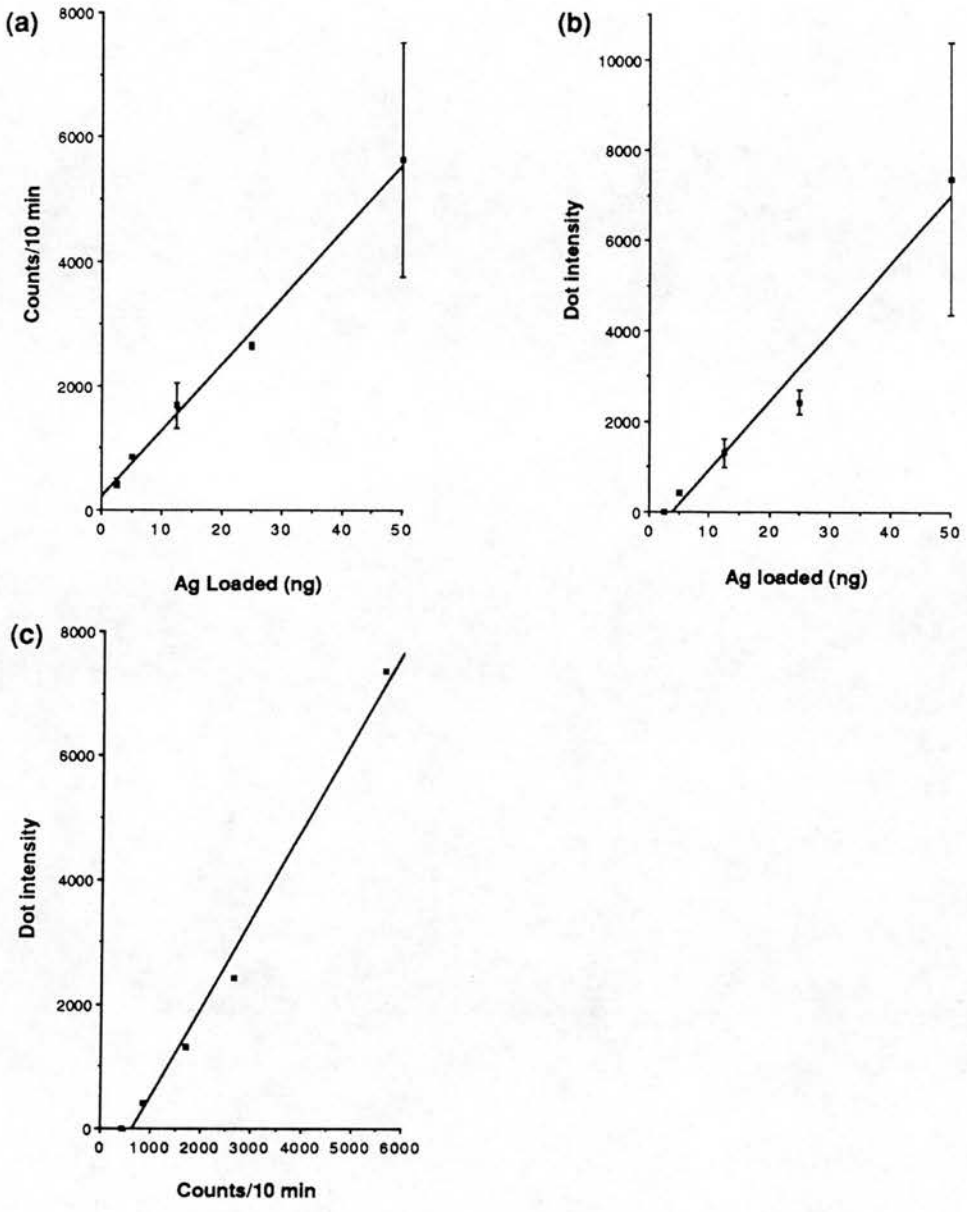
Linearity of the antibody-antigen reaction on dot blots.



Dot blots were carried out as described (Section 2.6.2) loading 250, 25, 2.5 and 0.25ng of each antigen in a volume of 1µl. Each blot was labelled with antiserum at a dilution of 1/500 and the antibody-antigen complexes detected by ^{125}I-protein A labelling as described in section 2.6.4. The squares were cut out, counted for 10min, and the specific binding of ^{125}I to each dot calculated as total counts bound minus counts bound to a duplicate blot incubated with pre-immune serum.

Figure 2.3.

Relationship between number of counts bound and dot intensity on dot blots.



A dot blot was carried out loading 50, 25,12.5, 5.0 and 2.5ng of purified PB_1 antigen in a volume of 1µl. The blot was labelled with anti-PB_1 antiserum at a dilution of 1/500 and the antibody-antigen complex detected by ^{125}I-protein A labelling as described. After exposure of the autoradiograph,the squares of nitrocellulose were cut out, counted for 10min, and the specific binding of ^{125}I to each dot calculated as total counts bound minus counts bound to a duplicate blot incubated with pre-immune serum. The intensity of dots on the autoradiograph was measured using a Joyce-Loebl Chromoscan 3 densitometer.
(a) number of counts/10min (mean ± SD). (b) dot intensity (mean ± SD).
(c) relationship between mean number of counts bound and mean dot intensity.

orientation markings made. The gel was discarded and the nitrocellulose filter washed twice (10 mins each) in 50mM Tris-HCl pH 7.9 containing 0.15M NaCl and 0.05% Tween 20 (TBST), then blocked in 3% low-fat milk powder in TBST for 1hour. All other washes were also of 10 mins duration and were carried out in TBST at room temperature with gentle shaking. After blocking, the filter was washed twice, incubated with the antiserum against the P-450 of interest (diluted 1/500 in TBST) for 1h, washed four times, incubated with horseradish peroxidase-conjugated goat antiserum raised against rabbit IgG (diluted 1/1000 in TBST) and washed again four times. It was then exposed to the horseradish peroxidase substrate 4-chloro-1-naphthol, which was made up as follows: 4-chloro-1-naphthol (120mg) was dissolved in 40ml of methanol and added to 200ml of TBS. Immediately before use, 80µl of 30% H_2O_2 was added. The filter was placed in this mixture and incubated at room temperature until bands became visible then washed briefly in distilled water. It was subsequently incubated with ^{125}I conjugated protein A (5 µCi in 50ml TBST) for 45 minutes and repeatedly washed with TBST until the radioactivity in the washes returned to background. The filter was air-dried overnight, taped to blotting paper, wrapped in clingfilm and exposed to Kodak XAR-5 film at -70°C in a cassette with intensifying screens. The film was developed after the necessary time (usually 4 - 48 hours for rodent liver microsomes) using a Fuji RG II X-ray film developer.

(b) Detection of P-450s in cell lines and human extrahepatic microsomes: Human tumour-derived cell lines and extrahepatic tissues express much lower levels of P-450s than does rodent liver. This caused a number of problems in the early stages of the project since the method described above was not sufficiently sensitive and specific to detect such low levels of P-450 expression without also labelling several other protein bands, presumed to be due to non-specific binding of the antiserum. A number of attempts were made to circumvent this problem; it was found that the most effective way to do so was to load the gel heavily, but to use more stringent blocking conditions to prevent non-specific binding. All blots on cell lines and human samples were therefore carried out in essentially the same way as those on rodent liver microsomal samples with the following modifications: 50µg of cellular protein was loaded into each track on the SDS-PAGE gel. The filter was blocked in 10% low-fat milk powder in TBST and all other washes and incubations (except for incubation with 4-chloro-1-naphthol) carried out in TBST containing 3% low-fat milk powder. This reduced non-specific binding to the extent that autoradiographs could be exposed for up to 3 months, greatly enhancing the sensitivity of the technique.

(c) Densitometric scanning of Western blots: In order to obtain an estimate of the relative amounts of a particular P-450 in different samples, some Western blots were further analysed by densitometric scanning using a Joyce-Loebl Chromoscan 3 densitometer. For this analysis, slightly underexposed autoradiographs were used in order to ensure that the film had not been saturated. The region of the band was scanned using a green filter with a scan length of 0.5 - 1cm and a slit width of 0.3mm. The relative intensity of each band was calculated as a percentage of that of a control band after checking that the intensities of the purified P-450 bands were linear with respect to the amount of protein loaded. It should be noted that although this precaution was taken this method is only semi-quantitative; small variations in protein loading, labelling and film background can significantly affect band intensity.
(d) Characteristics of the Western blot method: The labels used were chosen from those available (Moeremans et al (1984), Wilchek and Bayer (1984)) because staining with horseradish peroxidase and 4-chloro-1-naphthol allowed immediate visualisation of labelled bands whilst further labelling with ^{125}I-conjugated protein A increased versatility by allowing the length of the autoradiographic exposure to be adjusted as required. Short exposures could be made for densitometric scanning or long exposures for detection of low levels of antigen, whereas with horseradish peroxidase and 4-chloro-1-naphthol or other stains such as colloidal gold the staining intensity is limited by the chemical reaction. The limit of detection by Western blot analysis using horseradish peroxidase and 4-chloro-1- naphthol staining alone was approximately 10ng of purified P-450 antigen. Using the stringent blocking method for cell lines and human samples, 3ng of purified P-450 standard was easily detected and after long exposures lower levels could be detected on some blots. At this level of sensitivity there was some cross-reactivity between members of a sub-family (for example, it was extremely difficult to differentiate between the closely related proteins MC_{1a} and MC_{1b}), but the antibodies were readily able to distinguish between members of different gene families.

2.7. Enzyme assays.

2.7.1. Choice of assay methods.

In order to measure P-450 dependent activities in human cell lines it was necessary to use very sensitive and reproducible assay methods. Various methods are available; these were surveyed in order to identify an appropriate one for this project. Among

the P-450-dependent activities most commonly measured is the O-deethylation of 7-EC (Ullrich and Weber (1972)). The assay they described was relatively non-specific, since the activity was induced by both PB and 3-MC-type inducing agents, making it easy to screen samples for induction but difficult to identify the actual isozyme induced. The assay was insensitive because it involved direct measurement of the fluorescence of the reaction mixture, and interference was caused by turbidity and by NADPH, which fluoresces at the same wavelength range as the product, 7-hydroxycoumarin (7-OHC). Jacobson et al (1974) developed a method involving extraction with ether followed by thin layer chromatographic separation of 7-OHC from 7-EC which was sufficiently sensitive to allow them to measure 7-EC O-deethylase activity in human placental homogenates, whereas Greenlee and Poland (1978) used double extraction of 7-OHC into chloroform followed by 10mM NaOH to increase sensitivity. Further modifications were made to the assay to facilitate the measurement of 7-EC O-deethylase activity in cultured cells (Edwards et al (1984)). However, the necessity of using complicated extraction protocols before measuring the fluorescence of the product increased the possibility of error when working with samples having very low activities.

The reaction most commonly used to measure MC-type induction is the hydroxylation of benzo(a)pyrene (B(a)P), first described by Conney et al (1957). Over the following decade a number of groups modified the assay (Wattenberg et al (1962),Nebert and Gelboin (1968), Atlas et al (1976)) making it extremely sensitive. The reaction is usually quantified by extracting the products into an organic solvent (hexane or hexane/acetone) then into an aqueous alkaline solution (1M NaOH) and measuring its fluorescence under conditions optimal for 3-hydroxybenzo(a)pyrene (3-OHB(a)P) (excitation around 400nm, emission around 520nm). An assay in which the disappearance of B(a)P is measured directly has also been described (Yang and Kicha (1978)). The B(a)P hydroxylase assay, though well established, has a number of disadvantages. Like the 7-EC O-deethylase assay it involves a series of extractions, leading to error when assaying samples with low activities, especially since the extraction of 3-OHB(a)P is inefficient at low concentrations (Atlas et al (1976)). The reaction produces a large number of products, not all of which fluoresce under the alkaline conditions used for measurement of 3-OHB(a)P fluorescence (Holder et al (1975)), and this, combined with the ability of epoxide hydrolase to metabolise the reaction products, decreases

the sensitivity of the assay. Another major disadvantage of this assay is the carcinogenicity of the substrate, B(a)P.

A number of assays are available to measure the activities of PB-inducible P-450s, one of the most popular being N-demethylation of benzphetamine (Hewick and Fouts (1970)); others include N-demethylation of aminopyrine and ethylmorphine and oxidation of hexobarbital (Mazel (1971)). These assays involve repeated extraction steps or a secondary reaction to measure the formaldehyde produced (Nash (1953)), and have the disadvantage that the activities are only induced 2 -3.5 fold by PB and are relatively non-specific with regard to the isozyme involved (Lubet et al (1985a)). A more specific PB-inducible activity is the epoxidation of aldrin (Wolff (1980)); this requires gas-chromatographic analysis of the product, dieldrin.

2.7.2. Use of resorufin ether substrates to assess P-450 induction.

After comparing methods for measuring 7-EC O-deethylase and B(a)P hydroxylase with assays involving resorufin (phenoxazone) ethers it was decided to use the resorufin assays for the majority of this project. Over the past 15 years these assays, which utilise ether derivatives of the quinone resorufin, have proved to be among the easiest, quickest and most specific methods for measuring the induction of P-450-dependent activities. The first resorufin ether substrate to be used to assay P-450-dependent activity was 7-ethoxyresorufin (7-ER) (Burke and Mayer (1974)). This compound is suitable for measuring induction by compounds which act via the Ah-receptor since its metabolism is strongly induced in rat and mouse liver by MC though not by PB, correlates strongly with the expression of the major 3-MC-inducible P-450 (MC_{1b}) as detected spectrophotometrically, and is inhibited by alpha-naphthoflavone, B(a)P and antisera raised against isozyme MC_{1b} (Burke and Mayer (1974, 1975, 1983), Burke et al (1977, 1985)). The C_5 ether, 7-pentoxyresorufin (7-PR), was shown to be a good substrate for the major PB-inducible P-450 (PB_{3a}) since the activity is induced by PB and is inhibited by metyrapone, SKF 525A and antisera raised against isozyme PB_{3a},(Lubet et al (1985)). Studies using purified enzymes confirmed that isozyme MC_{1b} has high activity in 7-ER O-deethylation (EROD) whereas PB_{3a} metabolises 7-PR (Guengerich et al (1982a,b), Burke et al (1985), Wolf et al (1986)).

Examination of the metabolism of nine resorufin ethers showed that by using 7-ER

and 7-PR together with a third substrate, 7-benzyloxyresorufin (7-BR) it was possible to analyse the effects of different inducing agents on rat liver P-450s. The metabolism of 7-BR correlates most strongly with the induction of PB_{3a}, but this substrate is also metabolised by other P-450 isozymes (Burke et al (1985)). When used to measure microsomal metabolism, alkoxyresorufin dealkylase assays are carried out directly in the fluorimeter; they are rapid and the complete time course of the reaction may be examined without extracting the reaction product. These substrates are among the most selective of those available and are not known to be carcinogenic, making them very useful as probes for P-450 induction in microsomal samples. They have also been used to examine the spectrum of P-450 expression in extrahepatic tissues and in a variety of species (Rettie et al (1986), Mayer et al (1977)).

The resorufin ether assays quickly became established for measuring microsomal P-450-dependent metabolism, but problems arose in extending their use to 9000*g* supernatant fractions and tissue homogenates because a cytosolic enzyme appeared to reduce resorufin to a non-fluorescent secondary product (Nims and Lubet (1983)). Further studies on this phenomenon (Nims et al (1984)) suggested that it was due to cytosolic DT-diaphorase (quinone reductase) and that the reaction might be useful as an assay for DT-diaphorase. In order to allow the measurement of alkoxyresorufin dealkylation, the addition of 10μM dicumarol, a specific inhibitor of DT-diaphorase, to the reaction mixture, was recommended (Lubet et al (1985b)). An alternative method of avoiding this problem was to introduce a simple precipitation step into the method, using either methanol (Pohl and Fouts (1980)) or acetone (Grant et al (1988)) to remove the protein from the reaction mixture before measuring the fluorescence of the supernatant. This had the advantage of releasing protein-bound resorufin, thus increasing sensitivity, as well as denaturing DT-diaphorase and allowing the reduced resorufin to autooxidise. The method proved to be simple, sensitive, and reproducible and thus suitable for measuring P-450-dependent activity in human tumour-derived cell lines.

2.7.3. Methods used to assay alkoxyresorufin metabolism.

(a) Alkoxyresorufin metabolism in rodent liver microsomes (The direct method):

Alkoxyresorufin metabolism in rodent liver microsomes was assayed by a modification of the method of Burke and Mayer (1974). The method used was similar

for each of the alkoxyresorufin substrates used (7-ER, 7-PR and 7-BR). The assay was carried out directly in a Perkin-Elmer LS3 Fluorescence Spectrometer using a built-in temperature regulator set at 37°C. The reaction mixture consisted of substrate (1ml of 1μM in 100mM Tris-HCl pH 7.4, equilibrated to 37°C before use) plus sample (eg. 5μl of 10mg microsomal protein/ml for control rat liver microsomes or 10μl of 0.1mg/ml for MC-induced rat liver microsomes). These were mixed thoroughly in a 1ml glass fluorimeter cuvette and placed in the fluorimeter for about 1minute to check that the baseline was linear. The reaction was started by adding 10μl of NADPH (10mM in 100mM Tris-HCl pH 7.4). The fluorescence of the reaction mixture (excitation 530nm, emission 585nm) was monitored continuously on a Perkin-Elmer 561 Chart Recorder and the reaction was allowed to run until a measurable gradient was achieved. After about 3 minutes, 10μl of resorufin (10μM in ethanol) was added as a standard. Each assay was run in triplicate and the specific activities of the samples calculated as follows: The average pen deflection for a certain amount of standard added was measured and the deflection due to addition of 1nmol of resorufin calculated. The gradient for each sample could then be converted to specific activity in nanomoles of resorufin produced per milligram of microsomal protein using this value and the protein concentration of the sample. It was possible to make adjustments by altering the sensitivity setting on the fluorimeter and adjusting the amounts of sample and standard accordingly.

(b) Alkoxyresorufin metabolism in human tumour-derived cell lines (The acetone extraction method): Attempts to measure EROD according to the direct method of Lubet et al (1985b) were unsuccessful since dicumarol, at the concentration they recommended (which caused <20% inhibition of EROD activity in control and MC-treated rat liver microsomes) failed to prevent the NADPH-dependent loss of fluorescence in reaction mixtures containing NCI H322 cell sonicates. However, the method of Grant et al (1988) proved to be successful for measuring alkoxyresorufin dealkylase activities in a range of cell lines, and was therefore the method of choice for this project. Sonicated cell samples were diluted with 100mM sodium phosphate pH 7.6 to a protein concentration of approximately 1mg total cellular protein/ml and assayed under subdued yellow light in a photographic dark-room. The reaction was carried out in 5ml pyrex beakers using 3.08ml substrate (1μM in 100mM sodium phosphate pH 7.6) and 0.8ml sample. The components were mixed thoroughly and allowed to equilibrate at 37°C and the reaction started by adding 120μl of NADPH (10mM in 100mM sodium phosphate pH 7.6). A 500μl sample was taken every 5 minutes for 30 minutes. The reaction was quenched by adding the sample to 0.75ml

ice cold acetone in a microcentrifuge tube. After completion of the assay, samples were centrifuged at 14,000g for 5 minutes and the fluorescence of the supernatant (580nm/600nm) measured in a Perkin-Elmer LS3 Fluorescence Spectrometer. The reaction was standardised by incubating mock reaction mixtures containing substrate (1.6ml) plus control rat liver microsomes (400µl of 1mg microsomal protein/ml) in the absence of NADPH for 30 mins. After incubation a known amount of resorufin was added to each incubation mixture. Duplicate 500µl aliquots were treated in exactly the same way as the unknown samples. The rate of resorufin production was estimated by plotting the fluorescences of the extracts against time using the programme "Cricketgraph" on an Apple Macintosh computer. A straight line was fitted to each plot and the gradient converted to amount of resorufin produced using values obtained from the standard curve. The specific activity in picomoles of resorufin produced per milligram of total cellular protein was then calculated.

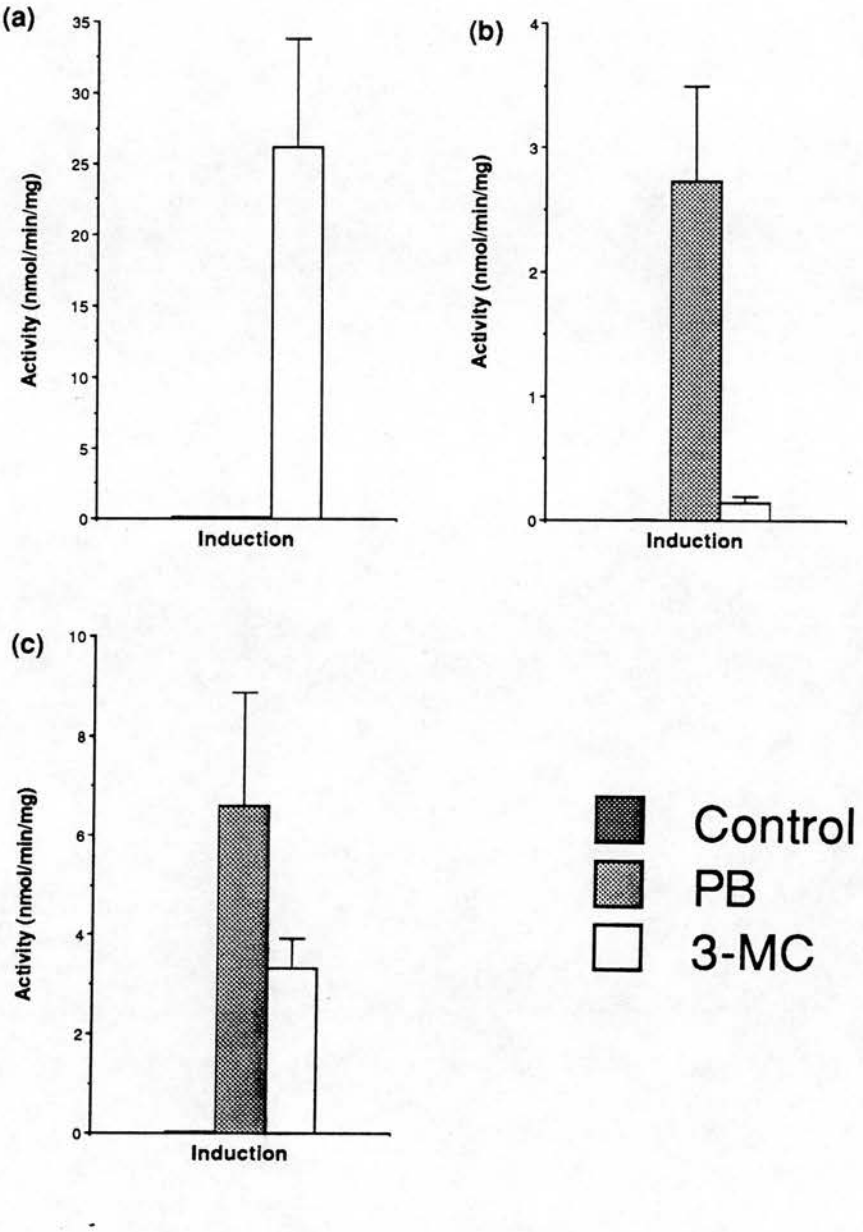
2.7.4. Characteristics of the alkoxyresorufin dealkylase assay.

(a) Reproducibility and pattern of induction in rat liver: In order to assess the reproducibility of the assay technique and confirm that the values obtained were in agreement with previously published results, the EROD, PROD and BROD assays were carried out by the direct method on the same pooled rat liver microsomal samples on five occasions over a period of six weeks (Figure 2.4). The results obtained correlated well with previously published findings (Burke and Mayer (1974), Lubet et al (1985a), Burke et al (1985)) although values were not always directly comparable since the temperature of incubation, strain of rat and induction protocol used varied between experiments. The results obtained in this experiment were generally reproducible for a given sample, but there was some day-to-day variation. The main source of error was the number of freeze-thaw cycles to which the samples had been subjected. In later assays it was ensured that enzyme assays were carried out the first time the samples were thawed, or on samples which had been through the same (small) number of freeze-thaw cycles. Another cause of variation was alteration of the fluorimeter sensitivity. The assay was most reproducible if the same sensitivity was used for all samples on a particular day, choosing the setting which gave a gradient of 10-50mm/min on the chart recorder for the least active sample and diluting the other samples in order to obtain a gradient within this range.

(b)Linearity of standards: The relationship between amount of resorufin added to the reaction mixture and fluorescence for both assay methods was shown to be linear over the range 0 - 1nmol resorufin/ml reaction mixture (Figure 2.5).

Figure 2.4.

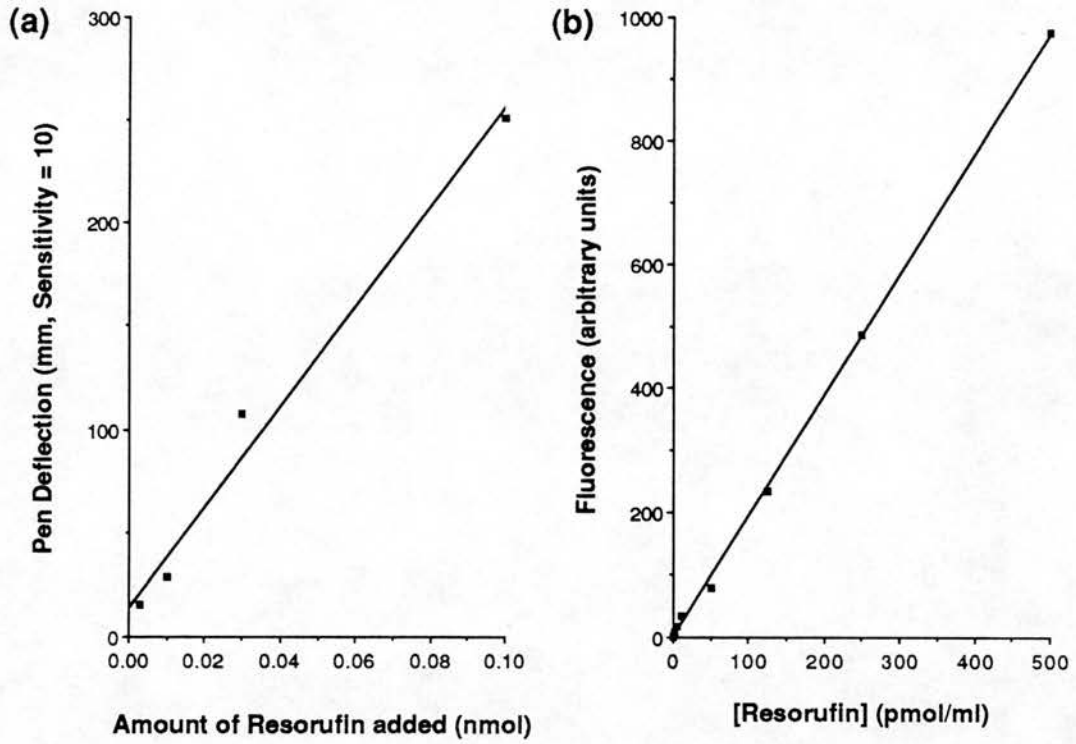
Induction of alkoxyresorufin metabolism in rat liver by phenobarbital and 3-methylcholanthrene.



Liver microsomes from control rats or rats treated with PB or 3-MC were pooled together and their activity towards three resorufin ether substrates assayed as described on five occasions over a period of six weeks. The results show mean ± SEM in nmol resorufin produced/mg microsomal protein/minute for five determinations.
(a) 7-ethoxyresorufin O-deethylase.
(b) 7-pentoxyresorufin O-depentylase.
(c) 7-benzyloxyresorufin O-dealkylase.

Figure 2.5.

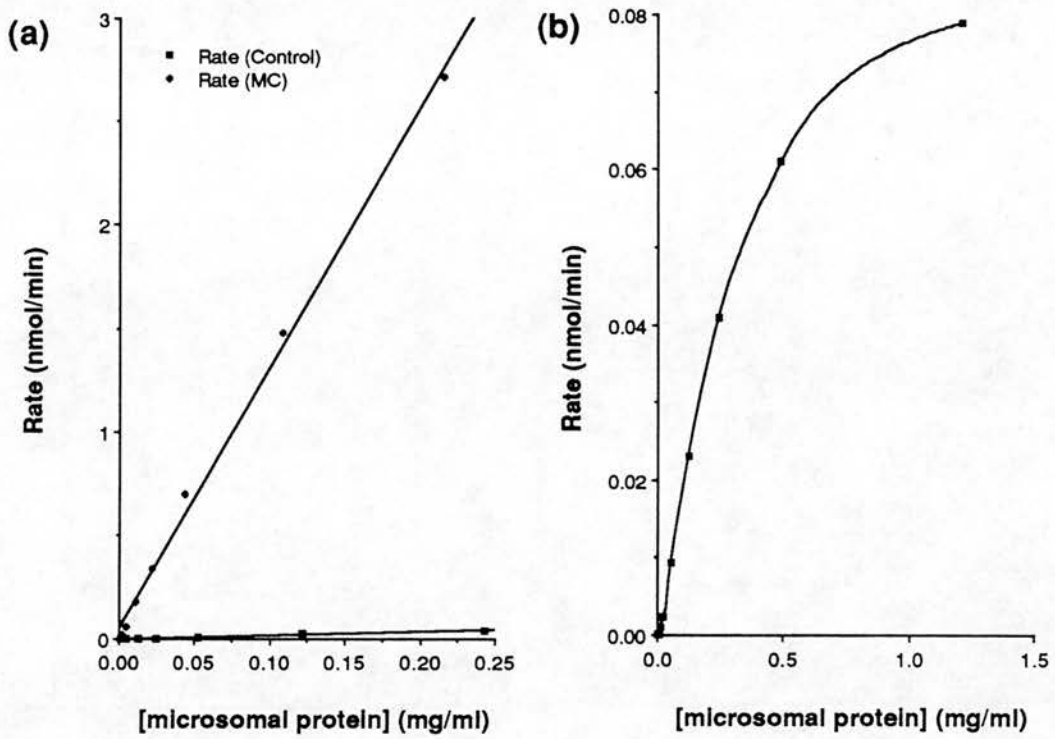
Relationship between resorufin concentration and fluorescence for two methods of carrying out the alkoxyresorufin dealkylase assay.



Standard curves over the range 0 - 100 pmol resorufin/ml in a background of 7-ethoxyresorufin were created using both the direct and the acetone extraction resorufin assay methods. A straight line was fitted through the points using the programme "Cricketgraph" on an Apple Macintosh Plus computer.
(a) Direct method.
(b) Acetone extraction method.

Figure 2.6.

Relationship between protein concentration and rate of reaction for the direct ethoxyresorufin O-deethylase assay.



In order to demonstrate the linear relationship between microsomal protein concentration and reaction rate for the EROD assay, the assay was carried out using a range of concentrations of pooled control and 3-MC treated rat liver microsomes and the rate measured in nmol resorufin produced/minute.
(a) linear region of the curve for control and 3-MC treated rat liver microsomes.
(b) effect of higher concentrations of control microsomes on the reaction rate.
High concentrations of 3-MC treated microsomes could not be used since the reaction rate obtained was too fast to measure accurately.

(c) Comparison of the two methods: The EROD activity of pooled control rat liver microsomes was assayed in quadruplicate by the acetone extraction method and the results compared with previous direct assays on aliquots from the same pool. Using the direct method the activity was estimated as 0.123 ± 0.038 nmol/min/mg microsomal protein; by the acetone extraction method the value obtained was 0.096 nmol/min/mg microsomal protein; this was within the range of previous estimates.
(d) Linearity with regard to protein concentration: The relationship between reaction rate and protein concentration was examined by the direct method using pooled control and MC-treated rat liver microsomes (Figure 2.6). The relationship was linear over the range 0.005 - 0.25 mg microsomal protein/ml, slightly lower than the range of 0.05 - 1.0 mg/ml reported by Burke and Mayer (1974)

2.8. RNA Analysis.

2.8.1. Preparation of total cellular RNA from cultured cells.

Total cellular RNA was prepared from confluent cells in 175cm^2 flasks according to the method of Birnboim (1988). All processes were carried out on ice using sterile diethylpyrocarbonate treated glassware, plastics and solutions. Gloves were worn to prevent contamination with RNAases. The culture medium was removed and the cells washed with PBS, then 4ml of RNA extraction solution (0.5M LiCl, 1M urea, 0.25% SDS, 0.02M sodium citrate, 2.5mM CDTA pH 6.8) was added and swirled gently until the monolayer lifted off the flask. The mixture was poured into a universal container and the flask washed with a further 2ml of RNA extraction solution which was added to the first wash. The sample was sonicated on low power for 10 seconds and treated with proteinase K (300µl, 20mg/ml) for 30 minutes at 50^{o}C. After the sample had cooled to room temperature 400µl of 2M sodium acetate pH 5.2 and 14ml of ethanol were added. The mixture was allowed to stand at -20^{o}C for 20 minutes then centrifuged (10,000rpm, 10 min) in a polypropylene tube. The supernatant was discarded and the pellet dissolved in 2ml RNA extraction solution and divided between two microcentrifuge tubes. Each was extracted with 100µl chloroform, vortexed thoroughly and centrifuged (13,000rpm, 5 minutes). The aqueous phase was transferred to a 2ml Sarstedt tube to which was added 7.5µl 2M acetic acid and 1ml LiCl/ethanol (5M LiCl: absolute ethanol, 3:2). The RNA was allowed to precipitate overnight at 4^{o}C and collected by centrifugation (13,000rpm, 2

minutes). The supernatant was discarded and the pellet dissolved in 200µl of 1mM sodium citrate, 1mM CDTA, 0.1% SDS pH 6.8). The quality and quantity of the RNA was assessed by measuring its OD_{260} and OD_{280}, since the ratio OD_{260}/OD_{280} for pure RNA is 2.0 whereas that for DNA is 1.8. The ratio OD_{260}/OD_{280} was >1.8 indicating that they contained little DNA. The concentration of RNA was estimated assuming that for a 1mg/ml solution of RNA, OD_{260} = 24.0; the yield of RNA was 700 ± 30µg per confluent 175cm^2 flask. The RNA was further assessed by running 5µg of each sample on a 1.5% agarose gel containing 7% formaldehyde. Samples were treated with three volumes of sample buffer (66% formamide, 8% formaldehyde, 1 x 4-morpholinepropanesulphonic acid) for 10 minutes at 50°C; 0.1 volumes of loading dye (15% Ficoll with bromophenol blue and xylene cyanol) were added and the samples loaded and run at 40V until the dye front neared the base of the gel. The gel was stained with ethidium bromide and examined using a UV Products Chromato-vue Transilluminator Model TM-40. Intact RNA samples contained two clear bands representing the major species of ribosomal RNA.

2.8.2. Northern blot analysis of cellular RNA.

Northern blot analysis was performed according to the method of Meehan et al (1984). The probe used was insert DNA from the plasmid pMP1, which contains a 1.1Kb fragment of MC_{1b} cDNA in the plasmid pUC9. The insert was isolated by digestion with the restriction enzyme EcoR1 followed by electrophoresis on low melting point agarose. Digestion was carried out in the following reaction mixture:

plasmid DNA	4µl (~4µg)
restriction enzyme buffer No. 3.	4µl
EcoR1 (10 units/µl)	1µl
sterile water	31µl

The reaction was allowed to proceed for 2 hours at 37°C then dye was added and the entire reaction mixture loaded onto a 1% low melting point agarose gel and run in TAE buffer (40mM Tris acetate + 1mM EDTA) until the dye front neared the base of the gel. The gel was stained with ethidium bromide, examined under ultraviolet light and the insert band (1.1Kb) cut out in 300mg of agarose. After addition of 450µl of water, the gel fragment was heated to 100°C for 7 minutes then equilibrated at 37°C for 10 minutes. Labelling was carried out by the random priming method (Maniatis et al (1982)). The reaction mixture was as follows:

sterile water	18µl
oligonucleotide labelling buffer	5µl
bovine serum albumin (1mg/ml)	2µl
insert DNA	20µl
alpha ^{32}P-dCTP (10µCi/µl)	3µl
Klenow fragment (1u/ml)	2µl

Oligonucleotide labelling buffer comprised Tris HCl (240mM), $MgCl_2$ (24mM), β-mercaptoethanol (0.4%) dATP, dGTP, dTTP (each 0.1mM), HEPES (1.0M) and hexadeoxyribonucleotides equivalent to 27 OD units/ml. The reaction was allowed to proceed overnight at room temperature. The next day, incorporation was assessed by chromatography on DE81 paper using 0.3M ammonium formate pH 8.0 as solvent. On an autoradiograph exposed for 20 minutes, insert DNA formed an intense spot at the origin whilst unincorporated nucleotides formed a faint smear above this point, indicating that incorporation of alpha ^{32}P-dCTP into the probe had occurred.

Samples of RNA (20µg) were run overnight at 40V on a 20 x 25 cm denaturing agarose gel as described above, washed for 10 minutes in 10 x SSC and transferred to Amersham Hybond N membrane by capillary transfer in 2 x SSC. Dilutions of SSC were made up from a 20 x SSC stock comprising 3M NaCl + 0.3M sodium citrate. The membrane was treated as follows: After transfer, orientation markings were made and it was washed in 2 x SSC, allowed to air-dry, and irradiated with ultraviolet light for 2.5 minutes, then baked for 1 hour at 80^{o}C. Prehybridisation was carried out in hybridisation mixture (5 x SSC, 4 x Denhardt's solution, 10% dextran sulphate, 0.1% SDS, 0.1% sodium pyrophosphate) for 1 hour at 68^{o}C in a sealed plastic bag. The probe was denatured by heating to 100^{o}C for 5 minutes then added to the bag containing the membrane and hybridisation mixture. Hybridisation occurred overnight at 68^{o}C, then the filter was washed three times at 68^{o}C in 2 x SSC containing 0.1% SDS and 0.1% sodium pyrophosphate, rinsed with 2 x SSC, wrapped in clingfilm and autoradiographed at -70^{o}C in a cassette with intensifying screens.

2.9. Cytotoxicity testing.

2.9.1. Choice of a cytotoxicity assay.

In order to assess the toxicity of P-450-inducing agents towards cultured cells it was necessary to choose a simple, rapid assay in which hydrophobic compounds such

as benzanthracene could be tested. A variety of assays for in vitro chemosensitivity, particularly to anticancer drugs, are available. Clonogenic assays have been widely used to assess the chemosensitivity of tumour cells in vitro (Puck and Marcus (1955), Hamburger and Salmon (1977)) and Courtenay and Mills (1978),. Other available assays range from those involving simple cell counts or dye exclusion techniques (Berry et al (1975), Weisenthal et al (1983b)) to those requiring sophisticated image analysis of crystal violet stained cells (Baker et al (1986)). Radiometric methods using parameters such as tritiated thymidine uptake, tritiated leucine labelling or $^{14}CO_2$ release from labelled glucose (Freshney et al (1975), Twentyman et al (1984), Von Hoff et al (1985)) are also commonly used. Clonogenic assays are considered to bear the most relevance to the behavior of tumour stem cells in vivo and have been subjected to detailed characterisation (Roed et al (1987)), but a variety of problems including low plating efficiency and clumping artifacts (Bertoncello et al (1982), Selby et al (1983)), as well as the time consuming nature of the assay, limit the usefulness of this method. The other assays are in general simpler to carry out, but there is considerable controversy about their comparability both with the clonogenic assay and with the behavior of tumour cells in vivo (Roper and Drewinko (1976), Weisenthal et al (1983a)). Recently, an assay relying on the ability of the mitochondrial enzyme succinate dehydrogenase in viable cells to cleave the ring structure of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) releasing a blue crystalline formazan product has been developed and adopted for the National Cancer Institute's anticancer drug screening programme. This assay is appropriate for both chemosensitivity and radiosensitivity testing (Carmichael et al (1987 a,b)). It appears to give results which are comparable to those of both the clonogenic assay and the dye exclusion assay of Weisenthal et al, although it cannot distinguish between cytostatis and cytotoxicity. The MTT assay was the assay of choice for testing the cytotoxicity of P-450-inducing agents, since it is rapid, simple, and may be manipulated in order to measure the toxicity of hydrophobic compounds.

2.9.2. The MTT assay method.

The MTT assay was carried out according to the method of Carmichael et al (1987a), which is itself a modification of the methods of Mosmann (1983) and Denizot and Lang (1986). Cytotoxicity assays were carried out using continuous exposure to the compound of interest. Cells were seeded at the required density (10,000 per well for

HepG2 or 2500 per well for NCI H322 in a volume of 180µl) in Costar 96-well microtitration plates. The outer wells of the plate were not used because evaporation tended to occur from these wells, but they were filled with medium to retard evaporation from the other wells. One column (6 wells) contained medium lacking cells as a blank. The plates were placed in a sandwich box lined with damp tissues to maintain high humidity and the cells allowed to adhere overnight. The next day, the compound to be tested was dissolved as a ten-fold stock in serum-free medium and serial one-in-two dilutions made. The compound was added to the wells in a volume of 20µl, each concentration being tested in triplicate and the blanks being treated with the highest concentration of test compound. After incubation for 4 -5 days at 37°C the plates were developed by adding 50µl of MTT (2mg/ml in distilled water, filter sterilised before use) to each well and incubating for a further 4 hours at 37°C. The medium was removed by aspiration with a Pasteur pipette and 50µl of DMSO added to dissolve the formazan crystals. The ODs of the wells were measured either at 570nm in a Dynatech MR 600 microplate reader or at 540nm in a Biorad Model 2550 EIA Reader. Spectral analysis of the formazan product (Table 2.4) showed that measurement of OD_{540nm} or OD_{570nm} gave directly comparable readings. None of the compounds used caused an increase in background optical density. The OD of each well was calculated as a percentage of the OD of wells containing cells in the absence of drug (% control OD) after subtraction of the blank. Each assay was carried out on at least three separate occasions and the results for each compound combined.

Table 2.4.

Spectral Characterisation of the MTT assay product.

	HepG2	**NCI H322**
Major peak	536.9nm	561.1nm
Minor péak	281.4nm	286.4
OD_{570nm}/OD_{max}	0.919	0.977
OD_{540nm}/OD_{max}	0.949	0.973
OD_{570nm}/OD_{540nm}	1.038	0.976

Confluent cells in a $25cm^2$ flask were treated with MTT (2.5ml in 12.5ml medium) as described. The medium was removed and the product dissolved in 2.5ml DMSO and diluted 1/20. Absorption spectra were read against DMSO in a Shimadzu MPS-2000 Multipurpose Recording Spectrophotometer over the range 280nm - 700nm.

The method described above was suitable for measuring the cytotoxicity of hydrophilic compounds such as phenobarbital and lymphokines. However, the technique had to be modified in order to assay hydrophobic P-450-inducing agents such as benzanthracene because it was not possible to acheive concentrations ten times those to which the cells were to be exposed, which were themselves close to saturation. The compounds were therefore dissolved in DMSO, the required dilutions made in growth medium, and this mixture used to feed the cells. On testing the toxicity of DMSO, it was found that 0.25% (v/v) was non-toxic towards both HepG2 and NCI H322 cells, so this was chosen to be the final DMSO concentration. A 400-fold stock of compound was made in DMSO and one in two serial dilutions made, also in DMSO. These were further diluted in medium containing serum (5µl drug/DMSO in 2ml medium). The medium was then aspirated from the cells and they were refed with fresh medium containing test compound. The remainder of the assay was carried out as described above. An extra control, cells exposed to 0.25% DMSO but not test compound, was introduced and the results were calculated as a percentage of the OD of this control. Using this method it was possible to test the cytotoxicity of a compound up to its saturation point if necessary.

2.10. Statistical analysis.

All experiments for which errors are shown were carried out at least three times (ie. on three or more mice or three cell culture experiments, using separate solutions as far as was practicable). Results shown are mean ± standard error of the mean, calculated using the programme "Statworks" on an Apple Macintosh Plus computer. Where analysis of statistical significance is shown, this was performed by Student's t-test (paired or unpaired, as stated) using the same programme.

Chapter 3.
Cytochrome P-450 expression in human tumour-derived cell lines.

3.1. Aims.

The aims of this section of the project were:
(i) to identify human lung- or colon-derived cell lines which express P-450s and are sensitive to P-450-inducing agents, in order to use them in studies of drug activation and regulation of P-450 expression by inflammatory mediators.
(ii) to compare the P-450 profiles of these cell lines with those of the human hepatoma-derived cell line HepG2 and human lung and colon tumour microsomes.

3.2. P-450 expression in short-term culture systems.

Various cell culture systems have been used for the study of P-450 expression and drug metabolism; this introduction will focus on those of rat, mouse and human origin. Cell culture systems may be divided into primary and continuous culture models; the characteristics of these models are described below.Hepatocytes are commonly used for studies of drug metabolising enzymes, since the liver is the major mammalian organ of xenobiotic metabolism. Both adult and foetal hepatocytes, which may be considered to represent an intermediate stage between primary and continuous culture cells, have been used in such studies.

3.2.1. Primary rat hepatocytes.

Primary adult rat hepatocytes isolated by collagenase perfusion and placed in short-term culture have been popular for drug metabolism studies for a number of years. However, the results of such studies were equivocal because the P-450 level

of primary hepatocytes was very low, even after treatment with inducing agents, and the values obtained were rarely compared directly with those of freshly isolated cells. It was found that P-450 levels and enzyme activities decreased rapidly over the first 1 - 2 days of culture (Guzelian and Bissell (1974), Guzelian et al (1977)), severely limiting the usefulness of primary hepatocytes as a model of in vivo P-450 regulation. The P-450 level fell to as little as one-tenth of the level found in freshly isolated cells by the tenth day in culture (Fahl et al (1979)). The situation was further complicated by the fact that P-450 isozymes did not decline at the same rate (Steward et al (1985)), and that reductase activity was maintained at about 50% of its in vivo level (Guzelian et al (1977)), so that the relation between P-450 and reductase levels differed from that observed in vivo. The mechanism of loss of P-450 expression is not understood; it was initially attributed to the concomitant increase in haem oxygenase activity which takes place over the first 24h of culture (Bissell et al (1974)). However, this hypothesis was later rejected because of the poor correlation observed between increased haem oxygenase expression and decreased P-450 levels (Paine and Legg (1978)).

Attempts to maintain P-450 levels in primary hepatocytes included growth of the cells on collagen membranes (Michalopoulos et al (1976a,b,c)). This method yielded healthier cells which retained some P-450 expression as well as the ability to respond to PB and 3-MC induction; however, the total P-450 level of these cells was low compared with that of freshly isolated cells. Patterns of PB induction reported in adult rat hepatocytes varied significantly: induction of P-450 by PB in cells grown on floating collagen membranes was reported, although the significance of this finding is debatable since a high level of the degradation product, P-420, was also observed (Michalopoulos et al (1976c)). In other studies, hepatocytes failed to respond to PB (Guzelian et al (1977)). These anomalies in PB inductibility compromise the use of primary adult rat hepatocytes as a model for studies of the mechanisms involved in P-450 induction. Further studies using coated tissue culture plates led to the development of a connective tissue biomatrix which enabled hepatocytes to retain differentiated functions for a number of months (Rojkind et al (1980), Schuetz et al (1988)). Hepatocytes co-cultured with rat liver epithelial cells apparently retained differentiated functions including secretion of albumin, P-450 expression, aminopyrine N-demethylase activity and susceptibility to the toxic effects of aflatoxin B_1 (Guguen-Guilluozo et al (1983), Begue et al (1984)).

Other studies demonstrated the importance of the culture medium in maintenance of P-450 expression. Supplementation of the hepatocyte growth medium with hormones or drugs such as metyrapone improved the maintenance of P-450 expression (Decad et al (1977), Paine et al (1982)). Metyrapone acts as a PB-like inducing agent in vivo; the higher level of P-450 in hepatocytes cultured in the presence of this drug may have been due to induction rather than maintenance of constitutively expressed P-450s. Recent studies emphasised the importance of medium composition in the maintenance of P-450 expression in culture (Grant et al (1985, 1986a). A modified Earle's medium was the most effective in maintaining P-450 levels in adult rat hepatocytes, with 63% of the P-450 content of freshly isolated hepatocytes being maintained in cells cultured in this medium for 48 hours. However, a steep decline in P-450-dependent enzyme activities was still observed; after 72 hours in culture the EROD activity of the cells had declined to 5% of that in freshly isolated hepatocytes. It was suggested that the high level of hydrocortisone in the modified Earle's medium might be important in its capacity to support P-450 expression. It appears that hepatocytes from different strains of rat have differing abilities to maintain P-450 expression in culture; a recent report indicated that hepatocytes from the Sprague-Dawley strain retained almost all their P-450 and associated activities during 72 hours in culture, whereas these parameters rapidly declined in hepatocytes taken from Hooded Lister rats (Grant et al (1986b))

There remain significant problems associated with the use of primary hepatocytes in studies on P-450 regulation. These include the requirement for large numbers of animals, variation between hepatocytes from different rats, and the absence of extrahepatic tissues which may affect drug responses. However, many valuable findings have been made using primary hepatocytes; for example, Guzelian and his colleagues demonstrated the de novo induction of P-450p (PB_{2C}) by pregnenolone-16α-carbonitrile (PCN) in primary adult rat hepatocytes (Elshourbagy et al (1981). Subsequent studies showed that dexamethasone was an even more efficient inducer of this isozyme and that the induction appeared to proceed via a non-classical receptor mechanism, unlike that of tyrosine aminotransferase (Schuetz et al (1984a,b)). This group also studied the effects of other inducing agents on isozyme P-450p and other drug metabolising enzymes, leading to the finding that P-450p may be "induced" both at the level of de novo synthesis and by specific inhibition of its degradation (Schuetz et al (1986a,b), Watkins et al (1986)).

3.2.2. Primary human hepatocytes.

Recent methodological developments have made it possible to prepare human hepatocytes and grow them in culture. Under certain conditions human hepatocytes retain a number of differentiated functions (Begue et al (1983), Clement et al (1984)), making it possible to study the pathways of human drug metabolism, including that of B(a)P and resorufin ethers (Monteith et al (1987), Grant et al (1987)). This model will prove valuable in comparing P-450 expression and regulation in animal models and humans.

3.2.3. Rodent foetal hepatocytes.

Unlike adult hepatocytes, foetal hepatocytes continue to divide in culture and may be sub-cultured by trypsinisation. These cells retain more P-450 expression than adult hepatocytes, but their properties differ significantly from those of adult cells; for example, higher levels of "MC-inducible" P-450 expression are observed, and this is further inducible not only by "MC-type inducing agents but also by PB (Gielen and Nebert (1971a), Goujon et al (1980)). Studies of primary foetal hamster cells (Gielen and Nebert (1971b, 1972)) revealed a similar pattern of regulation in this system. Significant differences in P-450 regulation exist between the foetal and adult hepatocyte systems, as well as between cultured hepatocytes and the liver in vivo, making the relevance of this system for studying P-450 regulation questionable. The initial advantage of foetal hepatocytes was that they continued to divide in culture, so that a higher yield of cells could be obtained from a single preparation: now that continuous-culture cell lines are available, foetal hepatocytes have been superceded as an experimental model.

3.2.4. Mitogen-stimulated human lymphocytes.

Mitogen-stimulated lymphocytes have also been used for the study of P-450 expression in humans. Measurement of lymphocyte AHH activities suggested that patients had one of three patterns of activity and response to TCDD ("low", "medium" and "high") which were related to suscepibility to lung cancer (Kellerman et al (1973)). Later studies did not support this conclusion, suggesting instead that the genetic regulation of AHH activity was polygenic and that both genetic and environmental factors were involved (Kouri et al (1974), Atlas et al (1976), Okuda et al (1977)). Kouri et al (1982) reported a good correlation between lymphocyte AHH activity and lung cancer incidence in smokers, but this observation

does not allow one to discern whether the high activity is a cause or an effect of lung cancer. Jaiswal et al (1985a) found that the increased level of MC_{1b} in lymphocytes correlated with AHH inducibility and Karki et al (1987) showed a correlation between lymphocte and lung AHH activity. Lymphocytes have the advantage that they are easily obtained and repeated samples can be taken from a single subject; however, their low levels of P-450 expression led to wide variability in experimental results. The entire body of information still does not permit definitive conclusions to be drawn about the relationship of the lymphocyte enzyme activity to cancer risk (Pelkonen et al (1984), Guengerich (1988)). It has been suggested that, since the lymphocytes have to be activated by mitogens before they can be cultured, variations in responsiveness to the mitogen, rather than to TCDD, are really being observed; this possibility has not been fully evaluated.

3.3. P-450 expression in continuously cultured cell lines.

As a result of recent advances P-450 expression can now be studied in continuously cultured cell lines. These have the advantage over primary cultured cells that many identical cultures may be produced over a period of time, improving reproducibility and allowing for long-term studies on P-450 regulation. Popular cell lines for drug metabolism work have been those derived from the liver, although extrahepatic cell lines which express P-450s have recently become available. P-450 expression has been studied in a variety of mouse and rat hepatoma-derived cell lines (Owens and Nebert (1975), Ferro et al (1984), McManus et al (1986)), but the most detailed studies have been carried out on rat cell lines derived from the Reuber H35 hepatoma and on the mouse cell line Hepa-1. These studies exemplify the usefulness of cell lines in extending our understanding of the mechanisms of P-450 regulation.

3.3.1. Reuber H35 hepatoma-derived cell lines.

Since being derived by Pitot (1964), the Reuber H35 rat hepatoma has given rise to many sublines, mostly derived from the cell line H4-II-EC_3 (Table 3.1). Their usefulness lies in the fact that they represent a range of phenotypes; they have been used in a series of studies to elucidate the importance of differentiation status in liver P-450 expression.

Table 3.1.

Cell lines derived from the Reuber H35 rat hepatoma line.

Cell line	Differentiation status	Relation to other lines
H35	Highly differentiated	Parent line
H4-II-EC_3	Lacks some liver-specific properties	Derived from H35
Fu-5	-	Derived from H4-II-EC_3
FAZA 967	Highly differentiated	Derived from Fu-5
H5	Poorly differentiated	" " "
Fao	Highly differentiated	Subline of FAZA 967
HF-1	Poorly differentiated	Hybrid between Fao and H5
HF1-4	Has some liver-specific properties	Subline of HF-1
C2Rev7	Highly differentiated	Derived from Fao
2sFou	Tetraploid	-
H4-II-EC3/G-	Selected in glucose-free medium	Derived from H4-II-EC3
Fu5-C8	Highly differentiated	Presumably from Fu-5
HF1.5	Highly differentiated	-

In 1974, Whitlock et al showed that AHH activity in H35 cells could be induced by concentrations of BA as low as 10^{-8}M, and that similar concentrations of Dex induce tyrosine aminotransferase. This was followed by the finding that PB and biogenic amines also induced AHH activity in these cells, suggesting that they behaved more like foetal than adult heptocytes (Owens and Nebert (1975)), which led to a series of studies on sublines of H4-II-EC_3. The more differentiated lines (FAZA 967 and Fao) were found to express detectable levels of aldrin epoxidase, a "PB-inducible" activity, and low levels of AHH, whereas the reverse was true in less differentiated lines (H5, HF-1). A subclone of the dedifferentiated line HF-1, HF1-4, which had regained some liver-specific properties, expressed both activities. Only the differentiated lines were inducible by Dex whereas BA was a better inducer in undifferentiated lines (Wiebel et al (1980)). Studies on the inducibility of the H4-II-EC_3 derived cell lines showed that in all the cell lines studied, PB induced AHH activity, but only in the differentiated lines could it also induce aldrin epoxide, confirming the conclusions of Owens and Nebert (Wiebel et al (1984a)). Antibody inhibition experiments showed that the cell lines could be classified into groups on

the basis of their P-450 profiles: those containing P-450 but not P-448-type cytochromes (differentiated lines, eg. C2Rev7), those containing only P-448-type cytochromes (poorly differentiated lines, eg. H5) and those containing members of both families (eg. 2SFou) (Wiebel et al (1984b)). These conclusions were recently confirmed by Northern blot analysis (Corcos and Weiss (1988)). Studies on other H35 hepatoma lines mostly agreed with these conclusions; however, the situation was complicated by the finding that, whilst the correlation between the state of differentiation of a cell line and its ability to respond to PB held good in most cases, one highly differentiated H4-II-EC_3-derived line, Fu5-C8, was refractory to PB-induction (Frey et al (1984)). Such studies illustrate the usefulness of such groups of cell lines in attempting to dissect the complex role of differentiation in controlling P-450 expression; studies using hormones to alter the differentiation status of these cells may help to improve our understanding of hepatic P-450 regulation.

3.3.2. The Hepa-1 mouse hepatoma cell line.

The cell line Hepa-1 has been of value in the elucidation of P-450 regulation via the Ah receptor. This cell line was shown to contain a functional Ah receptor which bound TCDD and other PAHs with high affinity (Kd = 0.45nM). As with steroid hormone receptors, circumstantial evidence indicated that translocation of ligand-receptor complexes from the cytosol to the nucleus occurred, though this hypothesis was not proven (Okey et al (1980)). Nuclear binding of the ligand-receptor complex led to transcription of a number of genes, including MC_{1b}. Studies on the Ah receptor in Hepa-1 cells showed that it had similar properties to the sodium-molybdate-stabilised glucocorticoid receptor, as does the Ah receptor of rat liver (Cuthill et al (1986), Wilhelmsson et al (1986)). Two groups carried out detailed studies on Hepa-1 variants with altered responsiveness to Ah-receptor ligands. Hankinson used a one-step selection procedure to isolate cells which were resistant to B(a)P toxicity (Hankinson (1979)). The cells were treated with BA (3μg/ml) for 18 hours in order to induce AHH activity, then exposed to 4μg/ml B(a)P. Cells in which induction of AHH activity occurred activated B(a)P to toxic derivatives and were killed, whereas cells in which induction had not occurred survived B(a)P treatment. In an alternative approach, Whitlock used fluorescence activated cell sorting to separate cells having different abilities to produce fluorescent metabolites from B(a)P (Miller and Whitlock (1981)). They used an induction procedure similar to that of Hankinson (3μg/ml BA for 16 hours) but then exposed the cells to a

non-toxic concentration of B(a)P, and assessed the accumulation of fluorescent products within the cells by fluorescence activated cell sorting. Complementation analysis allowed the resistant cell lines to be divided into three groups (Legraverend et al (1982), Miller et al (1983), Israel and Whitlock (1983)):

(A) those in which the Ah receptor appeared normal but the MC_{1b} structural gene was apparently mutated (Hepa-1c1c1, Hepa-1c1c3 and Hepa-1c1c5 of Hankinson)

(B) those which had a defective Ah receptor (Hepa-1c1c2 and Hepa-1c1c6 of Hankinson, BP^rc4 and $TAOBP^rc1$ of Whitlock)

(C) those in which the defect appeared to be in the nuclear binding of the Ah receptor (Hepa-1c1c4 of Hankinson, BP^rc1 and BP^rc3 of Whitlock)

Genetic analysis of type B and C variants and studies on nuclear association of the Ah receptor showed that the defects in ligand binding and chromatin binding were in different genes (Hankinson (1983), Whitlock and Galeazzi (1984)), leading to the conclusion that the Ah receptor in these cells consists of two separately encoded domains, whilst studies on Group A variants showed that both positive and negative regulation of MC_{1b} expression occurs (Hankinson et al (1985)). Whitlock's group used a variant having high B(a)P metabolising activity to isolate a regulatory domain of the MC_{1b} gene and to demonstrate the existence of a TCDD-responsive sequence and a putative repressor binding site in this region (Jones et al (1984, 1985)). They proposed that Ah receptor binding enhances expression by releasing the MC_{1b} gene from endogenous repression. An attempt was recently made to relate DNA methylation in the MC_{1b} gene to the regulation of its transcription (Peterson et al (1986)); no clear results emerged from this work and further studies are required to elucidate the mechanism of Ah receptor activity in this cell line.

3.3.3. Human tumour-derived cell lines.

AHH activity has been studied in various human cell lines, including the lung adeno-carcinoma line A549, the choriocarcinoma line JEG-3 and a number of squamous cell, Epstein Barr Virus-transformed B-cell and breast carcinoma lines (Fujino et al (1982), Hudson et al (1983), Wiebel et al (1984), Friedman et al (1985), Waithe et al (1986)). Activities in these cell lines were low but in most cases inducible by Ah receptor ligands such as BA or TCDD; in fact the MCF-7 cell line was used to clone and sequence a human MC_{1b} cDNA (Jaiswal et al (1985 a,b)).

Acetanilide-4- hydroxylase, AHH and MC_{1b} RNA in this cell line were all inducible

by TCDD (20nM) to about one-seventh of the levels observed in Hepa-1 cells, but surprisingly the Ah receptor was undetectable (Jaiswal et al (1985c)). Further studies with MCF-7, a TCDD-unresponsive sub-line MCF-7d_1 and HepG2 cells still failed to detect Ah receptors in MCF-7 cells (Cresteil et al (1987)). This paradox should soon be resolved now that TCDD-unresponsive sublines of MCF-7 are available (Cresteil et al (1987), Pasanen et al (1988)). In a recent study the Ah receptor was detected in the human squamous cell carcinoma line A413 (Harper et al (1988)), indicating that the problems in detecting the Ah receptor are not common to all human cell lines. Recent preliminary data indicating that a specific repressor of MC_{1b} expression exists in multi-drug resistant MCF-7 cells should open up new avenues of research into P-450 regulation in this cell line (Ivy et al (1988)) .

3.3.4. Cell lines studied in this project.

The aim of this project was to identify a lung- or colon-derived system in which to study P-450 expression. Four cell lines, two of lung origin and two of colon origin, were studied and compared with the hepatoma cell line HepG2 which is known to express cytochrome P-450s.

(a) HepG2: The human liver cell line HepG2 was derived from a childhood hepatoblastoma about ten years ago (Aden et al (1979), Knowles et al (1980)). Unlike most human hepatoma cell lines it retains a number of features characteristic of differentiated hepatocytes, including expression of apolipoproteins, blood clotting factors, complement components and growth factor carrier proteins (Zannis et al (1981), Saito et al (1982), Moses et al (1983), Goldberger et al (1984), Bahnak et al (1987)). As a consequence of these features, this cell line has been used to study aspects of liver metabolism including cholesterol homeostasis and iron uptake (Havekes et al (1983), Wu and Wu (1986)). Prior to this project, HepG2 cells were shown to be capable of the metabolic activation of drugs and carcinogens and to express some P-450-dependent activities (Dawson et al (1985)).

(b) NCI H322 and NCI H358: The human non-small cell lung carcinoma cell lines NCI H322 and NCI H358 are two of a large series of lung tumour cell lines derived by Minna and Gazdar as part of the National Cancer Institute's disease-oriented approach to the development of new anti-cancer drugs (Brower et al (1986)). The cell lines represent various types of lung tumours; ultrastructural analysis suggested that NCI H322 was of Clara cell type whereas NCI H358 was derived from the alveolar Type II pneumocyte (Schuller et al (1985)). These cell lines were

therefore of particular interest for this work because the Clara cell is thought to be the cell type which expresses the majority of lung P-450-dependent activity (Boyd (1977)), and the alveolar Type II pneumocyte, although it expresses less P-450 than the Clara cell, does have a significant level of P-450 expression. Both the Clara cell and the alveolar Type II pneumocyte are capable of the P-450-mediated metabolism of a number of model substrates including 7-EC, coumarin and B(a)P (Devereux (1984)) and are targets for toxic chemicals such as carbon tetrachloride and 4-ipomeanol which are activated by P-450s (Boyd et al (1980), Devereux et al (1982)). In rabbits treated in vivo with TCDD, these lung cell types respond with a 20 fold increase in the expression of MC_{1b} (Domin et al 1986)).

(c) HT29 and LS174T: HT29 is a colon adenocarcinoma cell line derived by Fogh and Trempe (1975)) which has been used for studies of intestinal functions such as those regulated by vasoactive intestinal peptide (Denis et al (1986), Bozou et al (1987)). This cell line is poorly differentiated under normal culture conditions but may be induced to differentiate by a number of exogenous factors such as poly-ethylene glycol (Laboisse et al (1988)). It was of interest to establish whether such a poorly differentiated line could be induced to express P-450s. LS174T is also a colon adenocarcinoma cell line, but it has a more differentiated phenotype than HT29. It was derived by Tom et al (1976) and retains a number of classical features of colon carcinomas including mucin secretion and expression of carcinoembryonic antigen (Hwang et al (1986), Miura and Kim (1986), Kuan et al (1987)).

3.4. Screening of cell lines for P-450 inducibility.

3.4.1. Dot blot analysis of P-450 induction in cell lines.

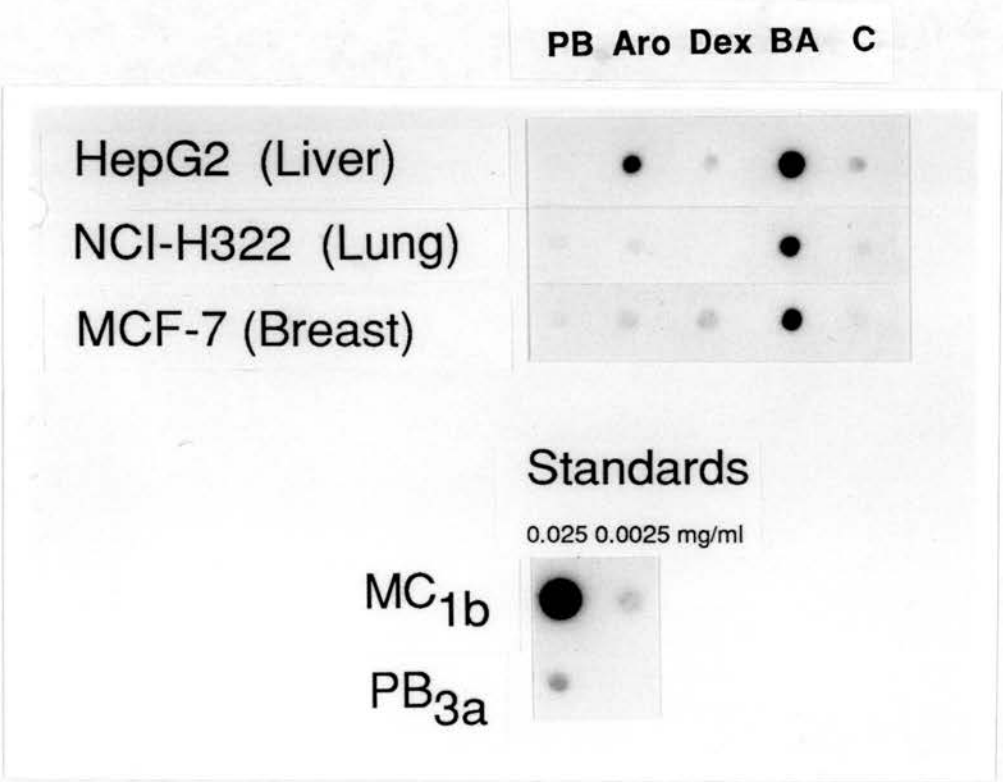
Preliminary screening for MC_{1b} induction in cell lines was carried out by dot blot analysis. Induction of MC_{1b} was observed in three cell lines, HepG2, MCF-7 and NCI H322 (Figure 3.1). Studies were continued using NCI H322 and HepG2 cells, together with other lung and colon tumour-derived cell lines.

3.4.2. P-450 induction in HepG2 cells.

The HepG2 cells initially tested were obtained from the Imperial Cancer Research Fund and grown in DMEM + 10% FCS. Western blot analysis of these cells (Figure 3.2) demonstrated that a protein which cross-reacted with the antiserum raised

Figure 3.1.

Dot blot analysis of isozyme MC_{1b} induction in HepG2, NCI H322 and MCF-7 cells.



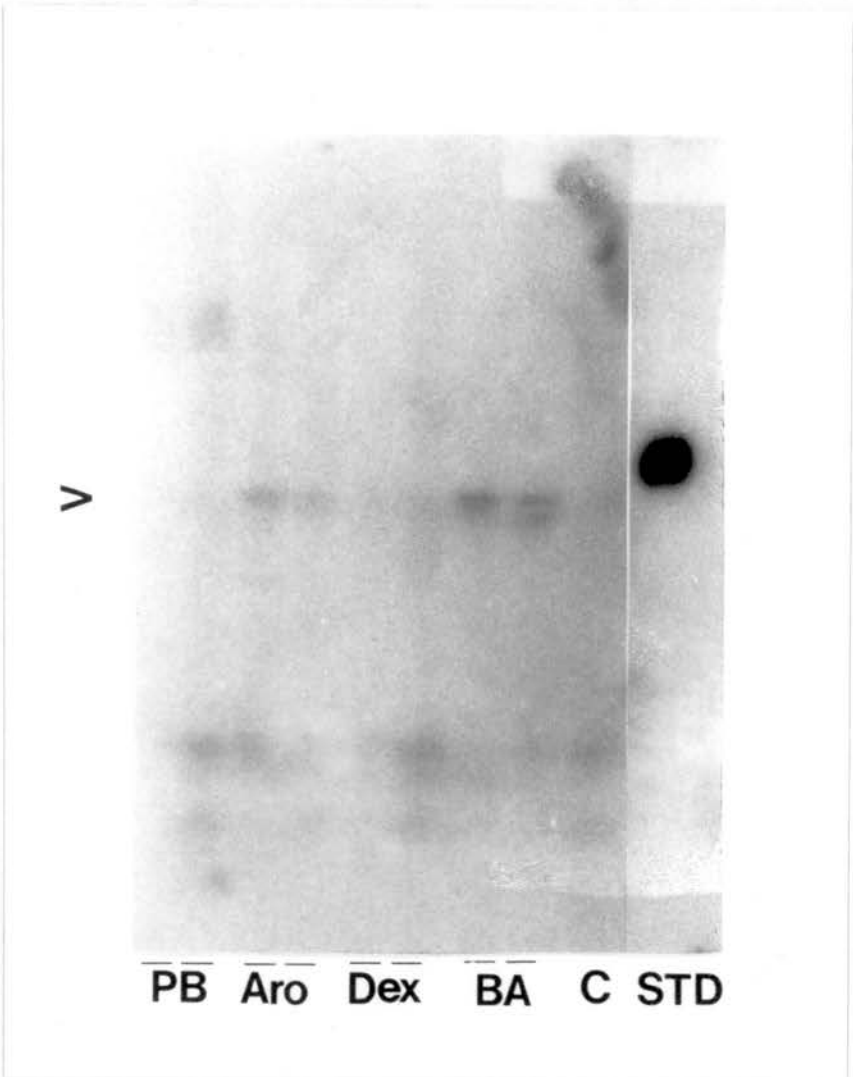
HepG2, NCI H322 and MCF-7 cells were grown to confluency in tissue culture flasks then treated as follows:

PB:	5mM phenobarbital for 48h.
Aro:	9µg/ml Aroclor 1254 for 24h.
Dex:	0.01mM dexamethasone for 48h.
BA:	9µg/ml 1,2-benzanthracene for 24h.
C:	Untreated control.

Sonicated whole cell samples (2.5µg protein) were dotted onto nitrocellulose, labelled with antiserum against isozyme MC_{1b} and autoradiographed after staining with ^{125}I-labelled protein A.

Figure 3.2.

Western blot analysis of isozyme MC_{1b} induction in HepG2 cells.



HepG2 cells were grown to confluency in tissue culture flasks then treated as follows:

PB:	5mM phenobarbital for 48h.
Aro:	3µg/ml Aroclor 1254 for 24h.
Dex:	0.01mM dexamethasone for 48h.
BA:	3µg/ml 1,2-benzanthracene for 24h.
C:	Untreated control.

Sonicated whole cell samples were prepared for SDS-PAGE and subjected to Western blotting as described, loading 100µg total cellular protein per track. The filters were labelled with antiserum against P-450 isozyme MC_{1b} and autoradiographed for 2 days after staining with ^{125}I-labelled protein A.

against isozyme MC_{1b} was induced strongly by BA treatment and to a smaller extent by Aroclor 1254. This protein had slightly faster mobility on the Western blot than the purified rat MC_{1b} standard. Problems were experienced with cells obtained from the ICRF because they grew slowly and failed to adhere to the culture surface if passaged more than three times after removal from liquid nitrogen. A new stock of HepG2 cells was obtained from Porton Down and grown in MEM + 15% FCS on the advice of the supplier. The inducibility of alkoxyresorufin metabolising activities in these cells is shown in Table 3.2. Basal EROD activity (5.71pmol/min/mg) in HepG2 cells was readily measurable; PROD and BROD were also detectable, though with more difficulty. The MC-like inducing agent BA induced EROD activity strongly (14 fold), and PROD and BROD activities slightly (3.5 and 5.2 fold, respectively). Western blot analysis confirmed that isozyme MC_{1b} had been induced in BA-treated cells; in this case induction by Aroclor 1254 was not detected.

Table 3.2.

Preliminary studies on the induction of alkoxyresorufin metabolism in HepG2 cells.

	Rate of Metabolism (pmol/min/mg):				
	Inducing agent:				
	Control	**PB**	**Dex**	**Aroclor**	**BA**
EROD	5.71	1.00	1.59	3.93	79.7
	(1.0)	(0.18)	(0.28)	(0.69)	(14.0)
PROD	0.028	0.002	0.004	0.018	0.098
	(1.0)	(0.07)	(0.14)	(0.64)	(3.5)
BROD	0.101	0.043	0.047	0.066	0.525
	(1.0)	(0.43)	(0.47)	(0.65)	(5.2)

Cells from the Porton Down Repository were treated with the following concentrations of inducing agents: PB - 4mM, Dex - 10μM, Aroclor 1254 - 3μg/ml, BA - 13μM (3μg/ml). All activities are given in pmol/min/mg total cellular protein and are the mean of duplicate determinations from two independent experiments. Values in brackets give the activities as fold induction compared with the control.

HepG2 cells have been grown in culture in various laboratories for approximately ten years, and during this time it is possible that phenotypic drift may have occurred, leading to differences in the behaviour of cells from different sources. Since HepG2 cells from two different sources were available the response of the two stocks to BA were compared. The cells were routinely grown under different conditions, so the effect of a change in conditions was also assessed (Table 3.3).

Table 3.3.

Comparison of EROD inducibility in HepG2 cells from different sources.

	Rate of Metabolism (pmol/min/mg):			
	Growth Medium:			
	DMEM + 10%FCS		**MEM + 15% FCS**	
	Control	**BA**	**Control**	**BA**
ICRF cells	11.0 (1.0)	86.8 (7.8)	5.0 (1.0)	87.8 (17.6)
Porton Down cells	43.3 (1.0)	107.8 (2.5)	8.0 (1.0)	142.0 (17.8)

Cells were treated with BA (13μM) or DMSO (Control). All activities are given in pmol/min/mg total cellular protein and are the mean of duplicate determinations from two independent experiments. Values in brackets give the activities as fold induction compared with the control.

The results of this analysis suggest that both the source from which the cells were obtained and the conditions under which they were grown affected their constitutive and inducible P-450 expression. The cells obtained from the Porton Down Repository grew more rapidly, adhered more strongly to the culture surface and generally appeared to be more healthy than those obtained from the ICRF. The Porton Down cells generally had higher P-450-dependent activities than those from the ICRF, the basal level, in particular, being higher if they were grown in DMEM + 10% FCS. A greater fold inducibility of cells grown in MEM + 15% FCS relative to those grown in DMEM + 10% FCS was observed, both because the BA-induced EROD activity of the former was higher and because their constitutive activity was lower.

3.4.3. P-450 induction in NCI H322 and NCI H358 cells.

BA was able to induce MC_{1b} in both NCI H322 and NCI H358 cells as assessed by Western blot analysis and at the enzymatic level (Figure 3.3, Table 3.4). The protein detected by Western blot analysis in BA-treated cells had slightly faster mobility than the purified rat MC_{1b} standard, as did that detected in HepG2 cells. Induction of this protein in NCI H322 cells was readily detected by Western blot analysis, but in NCI H358 cells only a very faint band was detected by this technique.

Table 3.4.

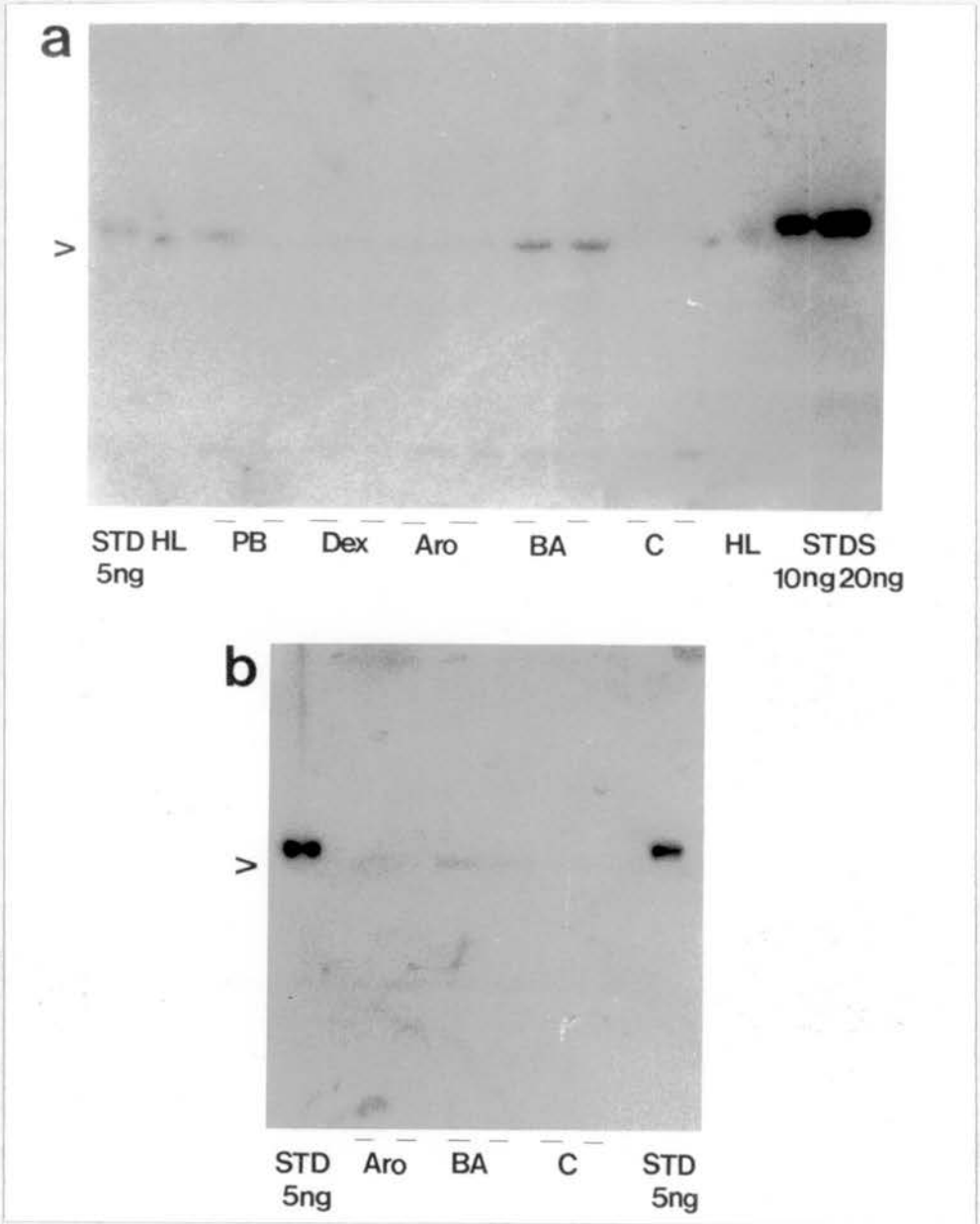
Induction of alkoxyresorufin metabolism in NCI H322 and NCI H358 cells.

	Rate of metabolism (pmol/min/mg):				
	Inducing agent:				
	Control	**PB**	**Dex**	**Aroclor**	**BA**
(a) NCI H322					
EROD	0.230	0.130	0.087	1.42	1.63
	(1.0)	(0.57)	(0.38)	(6.17)	(7.09)
PROD	0.019	0.031	0.015	0.013	0.021
	(1.0)	(1.63)	(0.79)	(0.68)	(1.11)
BROD	0.175	0.088	0.047	0.510	0.078
	(1.00)	(0.48)	(0.27)	(2.91)	(0.45)
(b) NCI H358					
EROD	0.123	-	-	0.243	1.37
	(1.0)	-	-	(1.98)	(11.15)
PROD	0.017	-	-	0.017	0.020
	(1.0)	-	-	(1.0)	(1.18)
BROD	0.059	-	-	0.098	0.147
	(1.0)	-	-	(1.66)	(2.49)

Cells were treated with the following concentrations of inducing agents: PB - 1 mM, Dex - 10 µM, Aroclor 1254 - 9µg/ml, BA - 40µM (9µg/ml). All activities are given in pmol/min/mg total cellular protein and are the mean of duplicate determinations from two independent experiments. Values in brackets give the activities as fold induction compared with the control.

Figure 3.3.

Western blot analysis of isozyme MC_{1b} induction in NCI H322 and NCI H358 cells.



NCI H322 and NCI H358 cells were grown to confluency in tissue culture flasks then treated as follows:

PB: 1mM phenobarbital for 48h.
Aro: 9µg/ml Aroclor 1254 for 24h.
Dex: 0.01mM dexamethasone for 48h.
BA: 9µg/ml 1,2-benzanthracene for 24h.
C: DMSO.

Sonicated whole cell samples were prepared for SDS-PAGE and subjected to Western blot analysis as described, loading 50µg total cellular protein per track. The filters were labelled with antiserum against isozyme MC_{1b} and autoradiographed after staining with ^{125}I-labelled protein A. The NCI H322 blot was exposed to film for 3 weeks and the NCI H358 blot for 2 months.

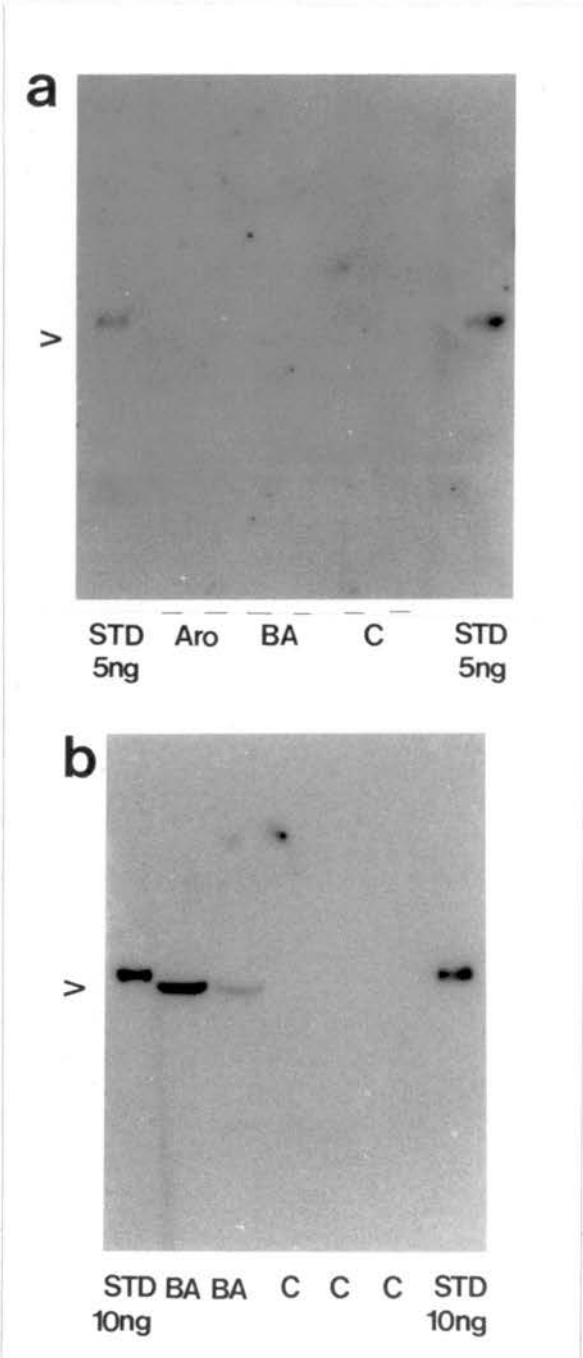
Table 3.4 shows that EROD activity was induced in both NCI H322 and NCI H358 cells (7 fold and 11 fold, respectively) by BA and that Aroclor 1254 also induced EROD and BROD activities (6 fold and 3 fold, respectively) in NCI H322 cells. The data suggest that at least two P-450 isozymes are inducible in NCI H322 cells, since BA induced only EROD activity whereas Aroclor 1254 induced both EROD and BROD activity. Further studies are required to identify the isozyme(s) induced by Aroclor 1254 since no induction by this agent was detected by Western blot analysis.

3.3.4. P-450 induction in HT29 and LS174T cells.

Induction of P-450s by BA and Aroclor 1254 was examined in the colon carcinoma cell lines HT29 and LS174T. Culture of LS174T cells for preliminary experiments was kindly carried out by Lesley Rooke in the Human Genetic Resources Laboratory, ICRF Laboratories, Clare Hall, South Mimms, Potter's Bar. Western blot analysis showed that MC_{1b} expression was barely detectable in HT29 cells, even after BA treatment, whereas a protein cross-reacting with antisera raised against this isozyme was readily detectable in LS174T cells following BA treatment (Figure 3.4). The protein detected by Western blot analysis had slightly faster mobility than the purified rat MC_{1b} standard, as did the proteins induced by BA in the lung and liver cell lines. One sample appeared to have a much higher induced level of this protein than the other; the reason for this anomaly is not clear, and further experiments were carried out (Section 3.5.1) to discover whether induction in this cell line was reproducible. Alkoxyresorufin assays were carried out in order to confirm the results obtained by Western blot analysis of HT29 and LS174T cells (Table 3.5). In agreement with the Western blot data, HT29 cells had very low P-450 activities, though their EROD activity was slightly induced by BA. In LS174T cells, on the other hand, this activity was strongly inducible by BA, the induced EROD activity of this cell line being as high as that of HepG2 cells.

Figure 3.4.

Western blot analysis of isozyme MC_{1b} induction in HT29 and LS174T cells.



HT29 and LS174T cells were grown to confluency in tissue culture flasks then treated as follows:

Aro: 5µg/ml Aroclor 1254 for 24h.
BA: 5µg/ml 1,2-benzanthracene for 24h.
C: DMSO.

Sonicated whole cell samples were prepared for SDS-PAGE and subjected to Western blot analysis as described, loading 50µg total cellular protein per track (standard 5ng) The filters were labelled with antiserum against isozyme MC_{1b} and autoradiographed after staining with ^{125}I-labelled protein A. The HT29 blot was exposed to film for 2 months and the LS174T blot for 1 week.

Table 3.5.

Induction of alkoxyresorufin metabolism in HT29 and LS174T cells.

	Rate of Metabolism (pmol/min/mg):		
	Inducing agent:		
	Control	**Aroclor**	**BA**
(a) HT29:			
EROD	0.080 (1.0)	0.134 (1.68)	0.266 (3.33)
PROD	0.002 (1.0)	0.012 (6.0)	0.007 (3.5)
BROD	0.017 (1.0)	0.065 (3.82)	0.056 (3.29)
(b) LS174T:			
EROD	0.234 (1.0)	- -	61.14 (261.3)
PROD	0.019 (1.0)	- -	0.010 (0.53)
BROD	0.114 (1.0)	- -	0.650 (5.7)

The concentrations of inducing agents used were: Aroclor 1254 - 5μg/ml, BA - 22μM (5μg/ml). All activities are given in pmol/min/mg total cellular protein, and are the mean of duplicate determinations from two independent experiments. Values in brackets give the activities as fold induction relative to the control.

3.5. P-450 expression in human lung and colon tumours.

3.5.1. Western blot analysis of P-450 expression in human lung and colon microsomal samples.

A number of normal and tumour samples from human lung and colon were available.* The clinical details concerning these samples are summarised in Table 3.6.

* See page 50 (footnote).

Table 3.6.

Clinical data concerning human tumour samples.

(a) Human Lung Samples

Code No.	Initials	Sex	Date resected	Tumour Type*	Differentiation
1	KP	F	22.03.83	Squamous	Poor
2	KH	M	08.03.83	Adenocarcinoma	Poor
3	JM	M	24.02.83	Large Cell	N K
4	LH	M	06.04.83	Small Cell	N K
5	AM	M	14.03.83	Squamous	Poor
6	RM	M	15.03.83	Squamous	Poor
7	TB	M	09.03.83	Adenosquamous	N K
8	MS	F	25.03.83	Squamous	Poor
9	AL	M	24.05.83	Squamous	Moderate
10	FB	M	01.03.83	Large Cell	N K

(b) Human Colon Samples

Code No.	Patient	Sex	Age	Duke's Status+	Location	Differentiation
1	87/2917	M	58	B	Rectum	Moderate
2	87/2936	M	51	A	Rectum	Moderate
3	87/5430	M	73	C	Sigmoid colon	Moderate
4	87/5576	M	64	A	Rectum	Moderate
5	87/5909	M	55	N K	N K	N K
6	87/7950	F	70	N K	N K	N K
7	87/9054	M	66	N K	N K	N K
8	87/9387	M	69	N K	N K	N K
9	87/9629	F	67	N K	N K	N K

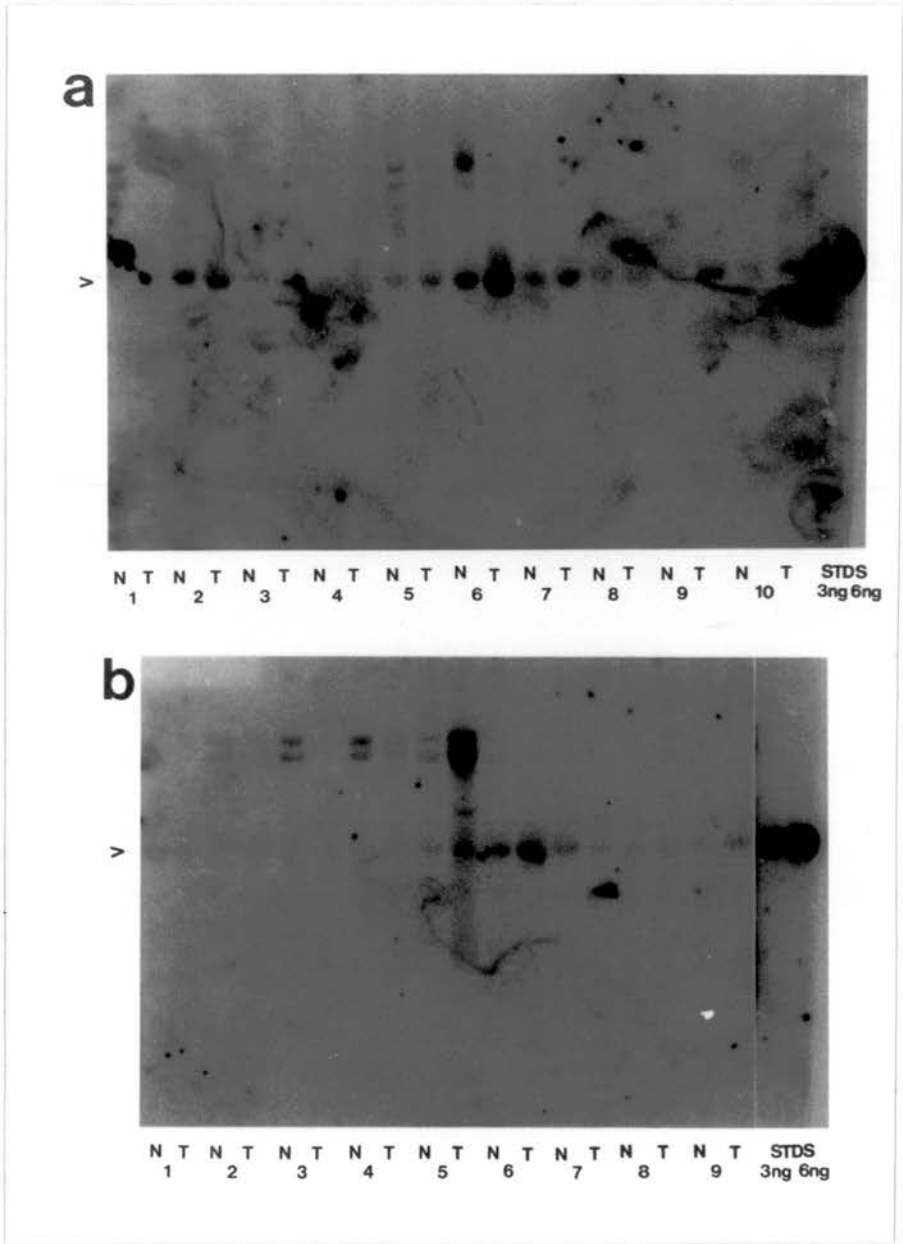
* Human lung tumours are classified histologically into a range of sub-types exhibiting variation in patterns of growth, response to treatment and prognosis (Monfardini et al (1987))
+The most commonly used system for classifying colon tumours and assessing their prognosis is that of Duke, summarised in Monfardini et al (1987).
N K - not known.

These samples were subjected to Western blot analysis using antisera raised against rat isozymes PB_1, PB_{2c}, PB_{3a} and MC_{1b} (Figures 3.5 - 3.8).

A band which cross-reacted with anti-PB_1 antiserum could be detected after 4 weeks exposure in all the human lung and colon samples (Figure 3.5). This band had the same mobility as the purified rat PB_1 standard and it is likely that it represents a true P-450 isozyme. However, some bands with high molecular weight also cross-reacted with this antiserum; the significance of these bands is not clear. The intensity of the band labelled in the P-450 region was variable in both lung and colon samples. In the lung most of the tumours had a more intense band than did the normal parenchymal samples; tumour No. 6 had a particularly intense band. In the colon samples there was no consistent relationship between normal and tumour levels of this protein, but one tumour (also No. 6) expressed an intense band cross-reacting with this anti- serum. Intriguing results were obtained in Western blot analysis using antiserum against rat isozyme PB_{2c} (Figure 3.6). Many of the lung samples contained two proteins which cross-reacted strongly with this antiserum. One band had a mobility similar to that of the purified rat standard whilst the other had slower mobility. The most striking finding was that lung tumour No. 6 had a very high level of both these proteins. In the colon samples, a faint band of the appropriate molecular weight was detected; the significance of this finding is hard to assess since many other proteins were also labelled on this blot. It was difficult to detect bands cross-reacting with antiserum raised against rat isozyme PB_{3a} in lung and colon samples (Figure 3.7). After lengthy exposure of the autoradiograph, faint bands having similar mobility to the purified rat PB_{3a} standard were detected; however, in both sets of samples a number of other proteins were also labelled and their significance is not clear. After such a long exposure it is difficult to distinguish between specific and non-specific binding of antiserum. The main finding emerging from this blot was that lung tumour No. 6 and colon tumour No. 6 appeared to contain more of this protein than did the other lung and colon samples. It was also difficult to detect isozyme MC_{1b} in the lung and colon samples (Figure 3.9). However, after lengthy exposure, proteins cross- reacting with antiserum against rat isozyme MC_{1b} could be detected in both lung and colon microsomes; a considerable amount of labelling of proteins outside the P-450 region of the blot occurred. Again, bands in lung tumour No. 6 and colon tumour No. 6 were labelled intensely. The band observed had a slightly faster mobility than that of the purified rat MC_{1b} standard, as did that of the human tumour-derived cell lines.

Figure 3.5.

Western blot analysis of isozyme PB_1 expression in human lung and colon microsomes.



Human lung and colon microsomes were prepared for SDS-PAGE and subjected to Western blot analysis as described, loading 30µg microsomal protein per track. The filters were labelled with antiserum against isozyme PB_1 and autoradiographed after staining with ^{125}I-labelled protein A. The human lung blot was exposed to film for 1 month and the human colon blot for 3 months.

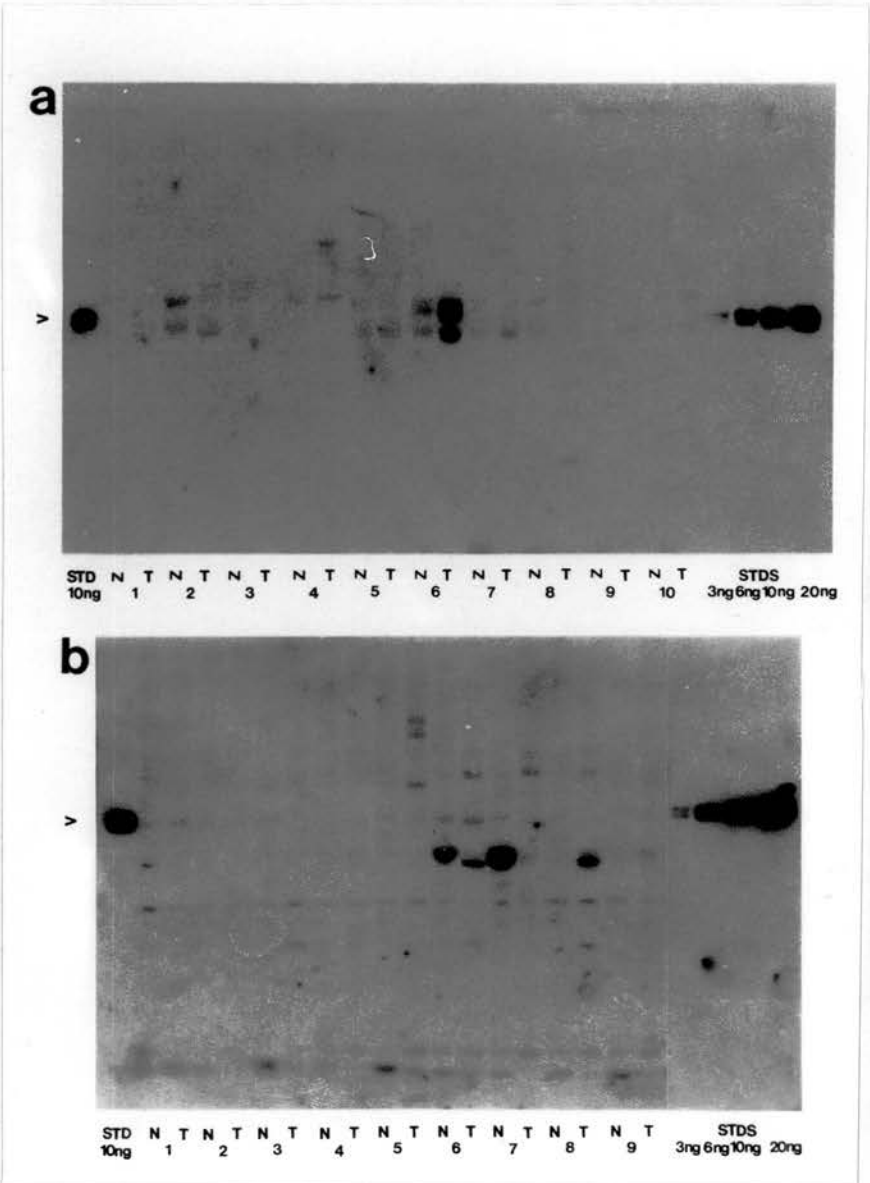
(a) Lung microsomes.
(b) Colon microsomes.

N = Non-tumour

T = Tumour

Figure 3.6.

Western blot analysis of isozyme PB_{2c} expression in human lung and colon microsomes.



Human lung and colon microsomes were prepared for SDS-PAGE and subjected to Western blot analysis as described, loading 30µg microsomal protein per track. The filters were labelled with antiserum against isozyme PB_{2c} and autoradiographed after staining with ^{125}I-labelled protein A. The human lung blot was exposed to film for 4 days and the human colon blot for 2 weeks.

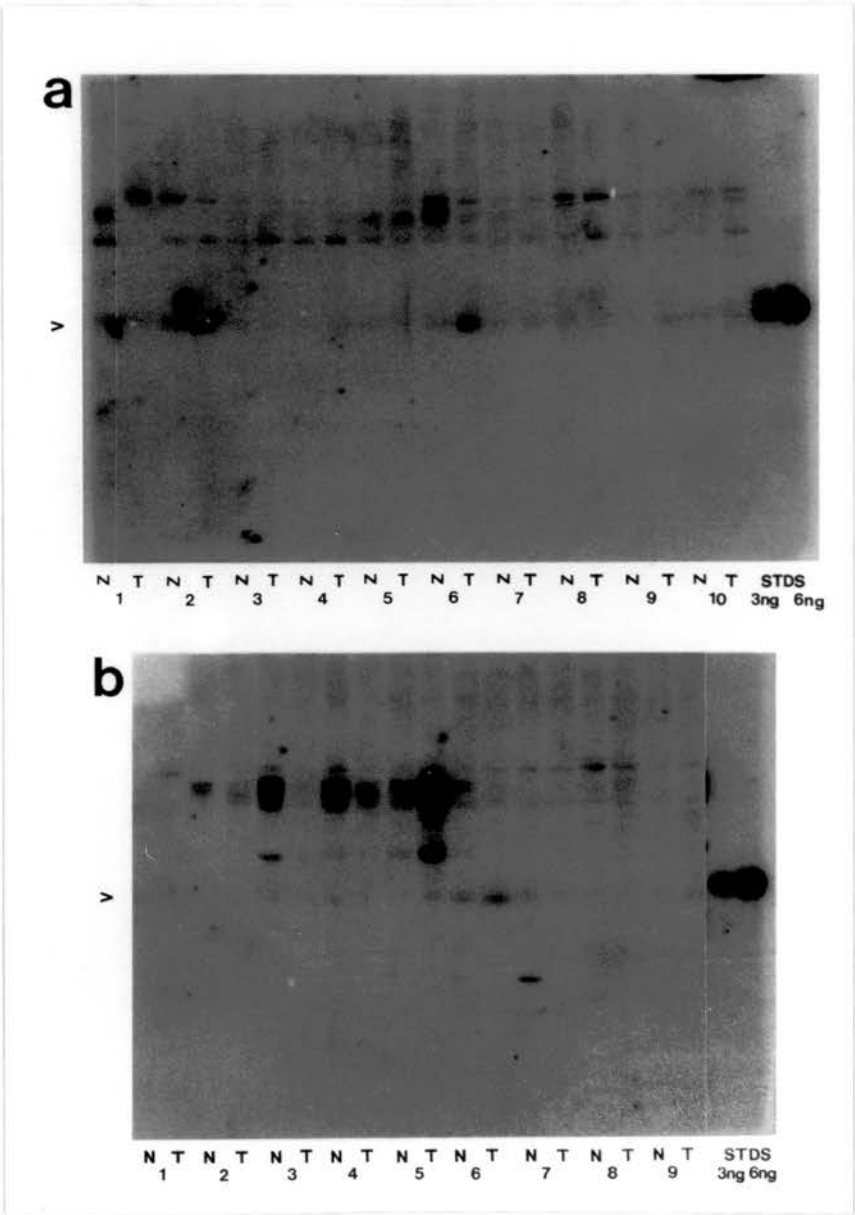
(a) Lung microsomes.
(b) Colon microsomes.

N = Non-tumour

T = Tumour

Figure 3.7.

Western blot analysis of isozyme PB_{3a} expression in human lung and colon microsomes.



Human lung and colon microsomes were prepared for SDS-PAGE and subjected to Western blot analysis as described, loading 30µg microsomal protein per track. The filters were labelled with antiserum against isozyme PB_{3a} and autoradiographed after staining with ^{125}I-labelled protein A. The blots were exposed to film for 3 months.

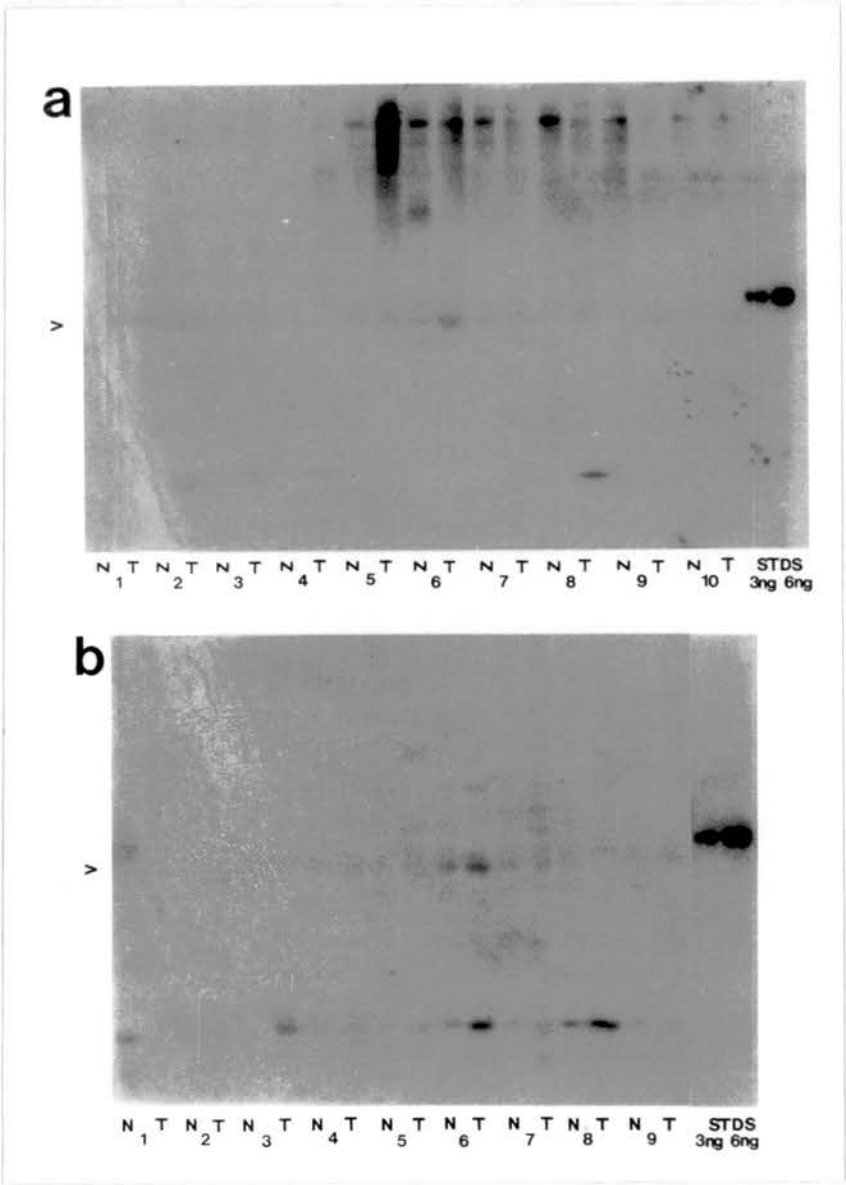
(a) Lung microsomes.
(b) Colon microsomes.

N = Non-tumour

T = Tumour

Figure 3.8.

Western blot analysis of isozyme MC_{1b} expression in human lung and colon microsomes.



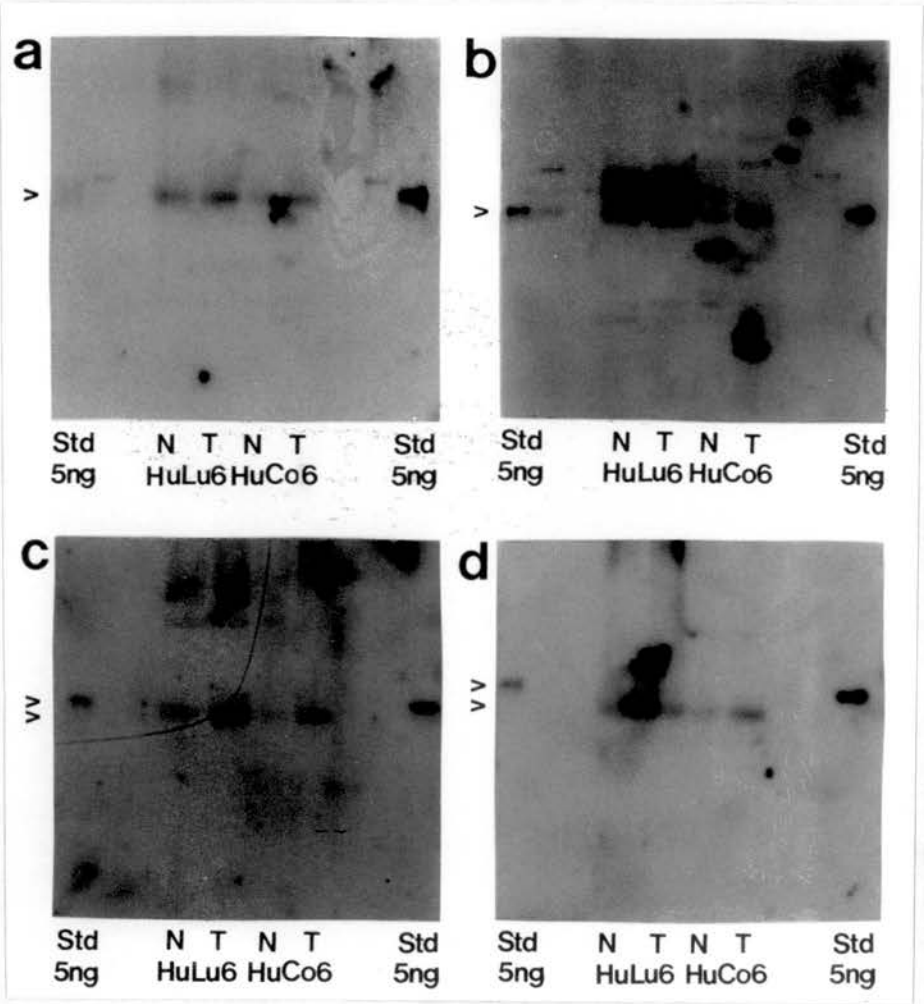
Human lung and colon microsomes were prepared for SDS-PAGE and subjected to Western blot analysis as described, loading 30µg microsomal protein per track. The filters were labelled with antiserum against isozyme MC_{1b} and autoradiographed after staining with ^{125}I-labelled protein A. Both blots were exposed to film for 3 months.
(a) Lung microsomes.
(b) Colon microsomes.

N = Non-tumour

T = Tumour

Figure 3.9.

Western blot analysis of the expression of P-450 isozymes in human lung No. 6 and human colon No. 6.



Western blot analysis of normal and tumour microsomes from human lung No. 6 and colon No. 6 was performed as described, loading 50μg of microsomal protein per track. The blots were labelled with antisera raised against the following P-450s

(a) PB_1

(b) PB_{2c}

(c) PB_{3a}

(d) MC_{1b}.

They were labelled with ^{125}I-labelled protein A and the autoradiographs were exposed for 2 months.

N = Non-tumour

T = Tumour

In order to confirm the expression of bands which cross-react with anti-P-450 antisera in human lung No. 6 and human colon No. 6, and to verify the mobilities of the labelled bands in these samples, Western blot analysis was repeated using each of the four antisera (Figure 3.9). The results indicated that the protein labelled using antiserum against PB_1 had the same mobility as the purified rat PB_1 standard. In the case of PB_{2c}, the faster of the two bands labelled had the same mobility as the purified rat PB_{2c} standard, whereas the other, more intensely labelled, band had substantially slower mobility. This band was greatly elevated in lung tumour No. 6. The bands labelled by antisera raised against isozymes PB_{3a} and MC_{1b} both had slightly faster mobility than the corresponding rat standards.

3.5.2. Alkoxyresorufin-metabolising activities in human lung and colon microsomal samples.

Assays of 7-ER, 7-PR and 7-BR metabolism were performed on the human lung and colon microsomes (Table 3.7). A single reaction mixture was used for each measurement because only a small amount of sample was available. The resorufin ether substrates were metabolised at very low rates by both normal and tumour microsomes. This study was, however, somewhat limited because the samples, particularly those from the lung, had been subjected to more than one freeze-thaw cycle before enzyme measurements were made. This may have been a cause of the very low activities which were detected. The EROD activities detected were about one thousandth of those previously reported in human liver microsomes (Adams et al (1985)). It is not possible to derive conclusions about the absolute levels of alkoxyresorufin metabolising activities in human lung and colon for this reason; however, a comparison of activities in normal and tumour material assayed on the same freeze-thaw cycle was made. Activity towards 7-ER was significantly reduced in the lung tumours relative to normal lung parenchyma from the same patients. As in other species (Rettie et al (1985)), human lung microsomes had higher activity towards BROD than towards other substrates. In the colon tumours, no significant difference in EROD activities between the normal and tumour material was observed; however, BROD activity in the colon samples was higher than activity towards the other substrates and was significantly lower in the tumours than in the normal material.

Table 3.7.

Metabolism of alkoxyresorufins by human normal and tumour lung and colon microsomes.

	Rate of Metabolism (pmol/min/mg):		
	Normal	**Tumour**	
(a) Lung samples			
EROD	0.092 ± 0.024 (0.019 - 0.254)	0.032 ± 0.011 (0.000 - 0.101)	T < N (p = 0.020)
PROD	0.079 ± 0.041 (0.000 - 0.440)	0.045 ± 0.010 (0.020 - 0.102)	NS
BROD	0.247 ± 0.162 (0.000 - 1.692)	0.187 ± 0.062 (0.000 - 0.480)	NS
(b) Colon samples			
EROD	0.053 ± 0.013 (0.002 - 0.109)	0.046 ± 0.006 (0.028 - 0.077)	NS
PROD	0.020 ± 0.006 (0.000 - 0.062)	0.016 ± 0.003 (0.008 - 0.034)	NS
BROD	0.230 ± 0.068 (0.000 - 0.700)	0.083 ± 0.027 (0.000 - 0.228)	T < N (p = 0.044)

All activities are given in pmol/min/mg microsomal protein and are the mean ± SEM for 9 or10 pairs of samples (i.e. normal and tumour samples from the same patient) for lung and colon data repectively. Values in brackets give the range of activities observed. The significance of the results was assessed using Student's paired t-test.

3.6. Characterisation of P-450 expression in NCI H322 and LS174T cells.

3.6.1. Reproducibility of induction.

The reproducibility of induction with Aroclor 1254 and BA in NCI H322 and

LS174T cells is shown in Figures 3.10 and 3.11. It was found that in NCI H322 cells induction of EROD and BROD activities was statistically significant ($p<0.01$ for induction of EROD by both BA and Aroclor 1254, $p<0.012$ for induction of BROD activity by Aroclor 1254). All three activities were reproducibly induced by BA in LS174T cells, and induction by BA, in particular, was highly significant ($p<0.001$). However, these cells were very difficult to grow as they often lifted off the growth surface before reaching confluency, in agreement with previous reports on their growth characteristics (Tom et al (1976)). The lung cell line NCI H322 was therefore chosen for subsequent studies on P-450 induction.

3.6.2. Northern blot analysis of MC_{1b} induction.

In order to discover whether P-450 induction was associated with increased steady-state mRNA levels, total cellular RNA was isolated from control and BA treated NCI H322 cells and subjected to Northern blot analysis using the 1.1Kb human MC_{1b} cDNA probe pMP1 (Figure 3.12). BA strongly induced an RNA fragment of approximately 3.0 Kb in these cells. The size of this RNA fragment is consistent with its being either the MC_{1a} message (3.1Kb (Quattrochi et al (1986)) or the MC_{1b} message (2.8Kb) (Jaiswal et al (1985a,b), Iverson et al (1987)). Further studies are required in order to identify the induced isozyme unequivocally.

3.6.3. Optimum concentration of benzanthracene for MC_{1b} induction.

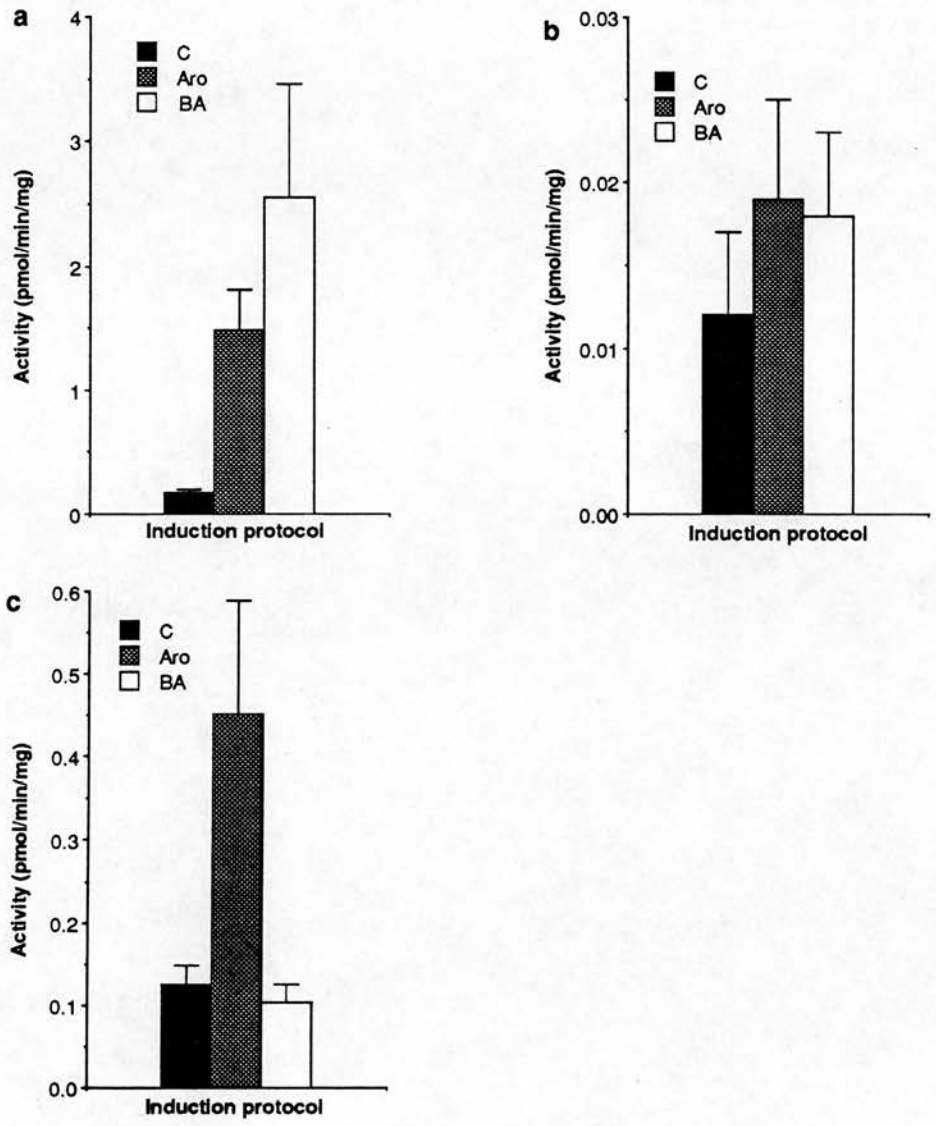
The effect of concentrations of BA in the range 0 - 30 μg/ml (0 - 130μM) on the induction process was studied (Figure 3.13). Induction of EROD activity was most marked at 3-5 μg/ml BA (13 - 22μM). On Western blot analysis (Figure 3.12b) the samples having the highest EROD activity contained a second protein which had faster mobility than the band previously observed in BA-treated NCI H322 cells. The significance of theis band is not known, but its level followed the same pattern as the metabolic activity towards 7-ER in these samples. Both proteins may contribute to BA-induced EROD activity in these cells.

3.6.4. Effects of different culture media on MC_{1b} induction.

The effects of three media, William's E, Minimal Essential Medium and RPMI medium, on the induction of MC_{1b} by BA were also compared (Figure 3.14). There were significant differences between the levels of EROD activity in both control and

Figure 3.10.

Induction of alkoxyresorufin-metabolising activities in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated as follows:

Control:	DMSO
Aro:	Aroclor 1254 - 9µg/ml
BA:	1,2-Benzanthracene - 9µg/ml

The cells were harvested and sonicated then frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The figures show mean activity ± SEM for at least 4 samples

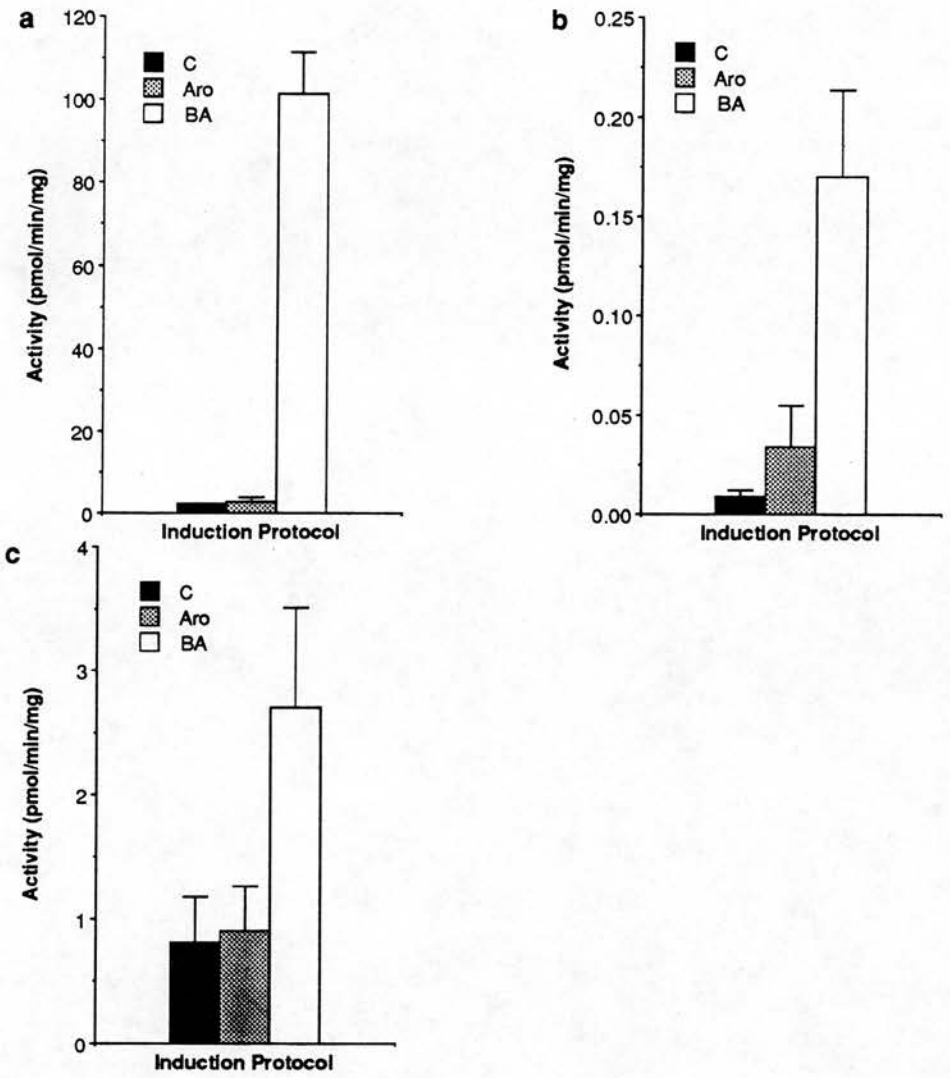
(a) EROD activity

(b) PROD activity

(c) BROD activity.

Figure 3.11.

Induction of alkoxyresorufin-metabolising activities in LS174T cells.



LS174T cells obtained from the European Cell Culture Repository at Porton Down were grown to confluency in tissue culture flasks using MEM + 10%FCS with non-essential amino acids. The cells were treated as follows:

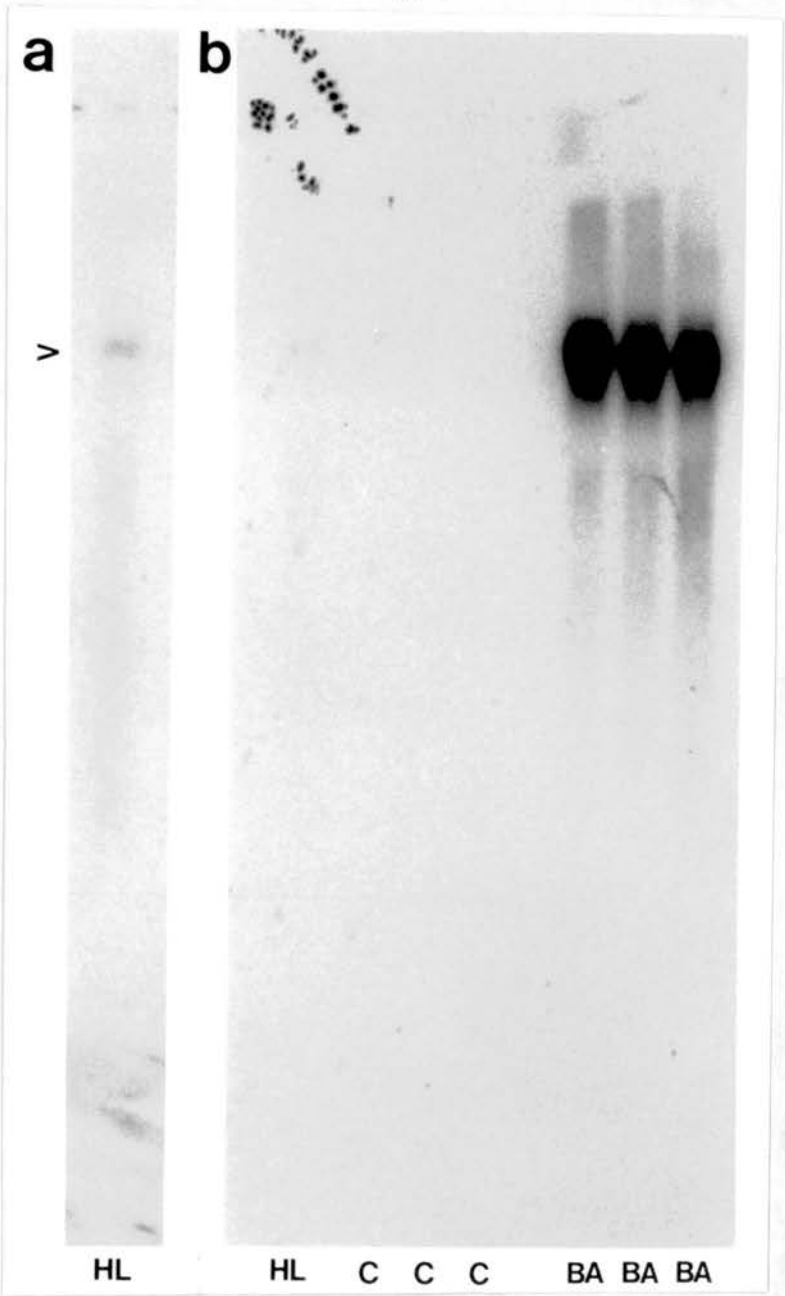
Control:	DMSO for 24 hours.
Aro:	Aroclor 1254 - 5μg/ml for 24 hours.
BA:	1,2-Benzanthracene - 5μg/ml for 24 hours.

The cells were harvested and sonicated then frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for at least 4 samples

(a) EROD activity
(b) PROD activity
(c) BROD activity.

Figure 3.12.

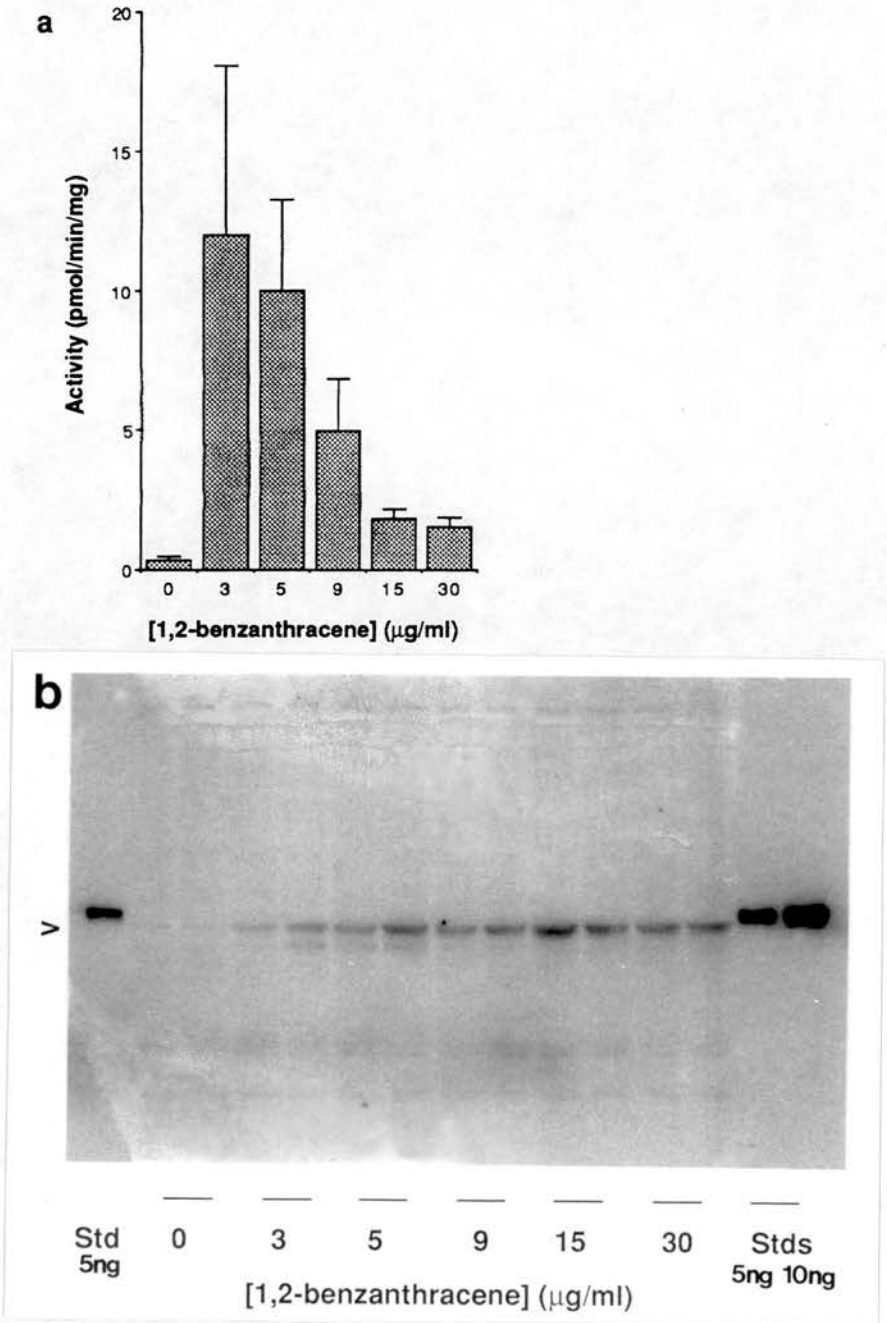
Northern blot analysis of MC_{1b} induction in NCI H322 cells.



NCI H322 cells were grown to confluency in 175cm^2 tissue culture flasks and treated with BA (5µg/ml) or DMSO (Control) for 24 hours. Total cellular RNA was isolated and subjected to Northern blot analysis as described, loading 20µg of RNA per track. 20µg of total cellular RNA from a human liver which was known to express MC_{1b} at a relatively high level was run in one track for comparison. The blot was labelled using the cDNA probe pMP1 and exposed to film for
(a) 1 week (to visualise the MC_{1b} band in the human liver sample).
(b) 40 hours.

Figure 3.13.

Effect of different concentrations of BA on MC_{1b} induction in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated with 0, 3, 5, 9, 15 or 30 μg/ml concentrations of BA for 24 hours. The cells were harvested and the extent of induction of isozyme MC_{1b} assessed by:

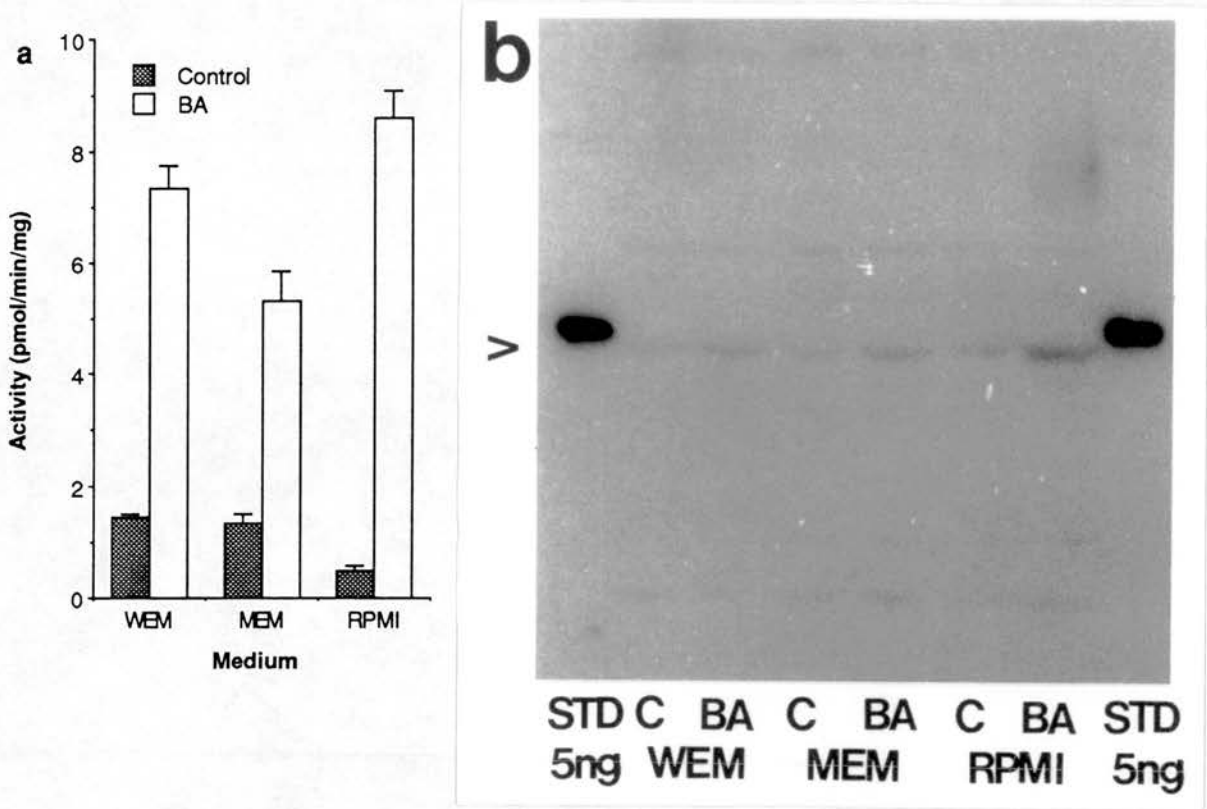
(a) Measurement of EROD activity

(b) Western blot analysis.

The EROD assay and Western blot analysis were carried out as described.

Figure 3.14.

Effects of different media on MC_{1b} induction in NCI H322 cells.



NCI H322 cells were plated out in tissue culture flasks in RPMI medium and allowed to adhere overnight. They were then refed with either WEM, MEM or RPMI medium and grown in these media for 8 days. After this time the cells were treated with BA (5μg/ml)or DMSO (Control) for 24 hours. The cells were harvested and the extent of induction of isozyme MC_{1b} assessed by:

(a) Measurement of EROD activity

(b) Western blot analysis.

The EROD assay and Western blot analysis were carried out as described.

BA treated cells. In control cells grown in RPMI, the constitutive level of EROD activity was only about one-third of that in cells grown in MEM or WEM ($p<0.01$), whereas the level of BA-induced activity in cells grown in RPMI was at least as high as that of cells grown in either of the other media. Cells grown in MEM had significantly lower BA-induced EROD activity than those grown in either of the other media ($p<0.04$). The cells in MEM grew more slowly than those in the other media and were subconfluent when harvested, so their lower EROD level may be related to their stage of growth. Growth cycle-related changes in EROD activity have been observed in HepG2 cells, cells in logarithmic growth having lower EROD activity than those which have reached confluency (Doostdar et al (1988)).

3.7. Discussion.

The data described in this chapter demonstrate that all five human tumour-derived cell lines studied had measurable levels of P-450-dependent activities; these activities varied widely from one cell line to another. The lung cell line NCI H322 and the colon cell line LS174T had high levels of BA-inducible EROD activities, those of LS174T being as high as those of HepG2 cells whilst those of NCI H322 were of the same order of magnitude but somewhat lower.

Dot and Western blot analysis showed that at least one protein immunochemically related to rat isozyme MC_{1b} was induced in HepG2 cells by BA. The induced protein had slightly faster mobility than the purified rat MC_{1b} standard. This finding agrees with the observation that the protein detected in human liver microsomes has faster mobility on Western blots than purified rat MC_{1b} (L.M.Forrester, personal communication), and with reports of a 52,500d protein in human liver which cross-reacts with antisera raised against rat MC_{1b} (Wrighton et al (1986)). The molecular weight of rat MC_{1b} itself is reported to be 53,500 - 56,000d (Lu and West (1980)). Some antisera raised against rat MC_{1a} or MC_{1b} detect a second protein of approximately 57,000d in human liver microsomes (Adams et al (1985), L.M.Forrester, personal communication). Two bands were detected in BA-treated HepG2 cell samples, but both had faster mobility than the purified rat MC_{1b} standard and neither was likely to be equivalent to this 57,000d protein. The detection of alkoxyresorufin activities in the hepatoma cell line HepG2 is in

agreement with previous work in this laboratory (Dawson et al (1985)) which showed that ethoxycoumarin O-deethylase activity in HepG2 cells was induced by MC (5μM). However, in that study no MC-inducible proteins were detected at the Western blot level. The improved sensitivity of the Western blot method used in this study made it possible to detect the induction of MC_{1b} immunochemically as well as enzymatically after BA treatment. HepG2 cells have recently been shown to metabolise various xenobiotics by both P-450-mediated and other pathways (Bhatt (1986), Sassa et al (1987), Duthie and Grant (1988)). The most detailed studies have been those carried out by Grant et al (1988) demonstrating that HepG2 cells express alkoxyresorufin and UDP-glucuronyl transferase activities, and that EROD activity in these cells is induced 15 fold in response to BA (25μM). The present results are in good agreement with these data. It has also been shown that the growth medium used to culture the cells is important in the control of P-450 expression in both HepG2 cells and primary human hepatocytes (Grant et al (1987), Doostdar et al (1988)). It is clear that HepG2 cells have the essential mechanisms for the study of Ah induction; they are currently being used to study various aspects of P-450 regulation such as Ah-receptor activity (Roberts et al (1987)) and the 5' regulatory sequences of the human MC_{1a} and MC_{1b} genes (Quattrochi et al (1988)). Work has also been progressing on the maintenance of glutathione balance in HepG2 cells (Duthie et al (1988), Tate and Galbraith (1988)), further extending the value of this cell line for the study of human drug metabolism.

The finding that the human lung tumour-derived cell lines NCI H322 and NCI H358 express EROD activity which can be induced by BA correlates with the studies of Falzon et al (1986a) who showed that both cell lines express BA-inducible AHH activity, though NCI H358 has slightly lower activity and inducibility than NCI H322. These cell lines are able to activate the pulmonary toxins 4-ipomeanol and N-nitrosodiethylamine (McMahon et al (1985), Falzon et al (1986b,c)); they also express several conjugating enzymes (Wiebel et al (1986)) as well as enzymes involved in arachidonic acid metabolism (Lau et al (1987)). These findings, together with those described above, suggest that NCI H322 cells, in particular, represent a good model for the study of drug metabolism in the human lung.

No previous studies have been reported on the induction of drug metabolising enzymes in human colon tumour-derived cell lines. The finding that LS174T cells

express high levels of EROD activity in response to BA is therefore of great importance, and implies that further studies should be carried out using this cell line. The low activities detected in HT29 cells were not surprising in view of their undifferentiated phenotype, and this illustrates the wide variability of P-450 levels even in cell lines derived from the same tumour type. These colon cell lines represent a potentially useful model for P-450 regulation in human colon. LS174T cells, whose EROD activity has been shown above to be highly inducible by BA, normally consist of a mixed population of mucin-secreting and non-mucin-secreting cells, but these may be separated by a replica plating technique (Kuan et al (1987)). Since colon tumours which produce large amounts of mucin have a poor prognosis, studies on P-450 expression in the two types of LS174T cells might clarify the role of drug metabolising enzymes in this phenomenon. LS174T cells may also be induced to undergo further differentiation by treatment with sodium butyrate (Gum et al (1987)) and the use of this system would help to establish the role of differentiation in colon P-450 regulation. Similarly, although HT29 cells are normally undifferentiated in culture, certain subclones of this cell line may be induced to differentiate, for example by growth on permeable filters or treatment with polyethylene glycol (Laboisse et al (1988), Phillips et al (1988)). It would be very interesting to see whether P-450 inducibility in this cell line was restored as a result of growth under conditions which favour differentiation.

The cell lines NCI H322 and LS174T were chosen for further study. They were first compared with normal and tumour microsomes from human lung and colon, which were analysed by Western blot analysis and by measuring their alkoxyresorufin metabolising activities. Proteins cross-reacting with all four of the antisera used (raised against isozymes PB_1, PB_{2c},PB_{3a} and MC_{1b}) could be detected in human lung and colon samples. Long autoradiographic exposures were required in order to detect these proteins, and the significance of bands detected in the P-450 region of the blot was hard to assess since a considerable amount of labelling of bands having molecular weights well outside this region was also detected. However, the results will be discussed making the assumption that the bands having the closest mobility to that of the rat standards represent true P-450s. The detection of four P-450s, PB_1, PB_{2c}, PB_{3a} and MC_{1b}, albeit at low levels, in the human lung and colon microsomes contrasted with the results obtained with cell lines, in which P-450s could not be detected in uninduced cells. Indeed, only isozyme MC_{1b} was detected at all in cell

lines, and then only after BA treatment. This may represent a real difference in the expression of P-450s between human lung and colon and cell lines derived from these tissues; alternatively, this finding may be due to the fact that microsomes were prepared from the solid tissue samples whereas whole cell sonicates from the cultured cells were used. The level of P-450 expression detected was very low and the ability to detect it may have been a result of concentration of P-450s in the microsomal samples compared with whole cell sonicates.

The isozyme most consistently detected in the human lung and colon samples was PB_1, which was present at variable levels in all the samples studied. The expression of proteins immunochemically related to PB_{2c}, on the other hand, varied greatly from one sample to another. One lung tumour, No. 6, contained an exceptionally high level of two such proteins, one of which had the same mobility on Western blots as purified rat PB_{2c} whereas the other had somewhat slower mobility. This is not the first time that a slowly migrating band has been detected in material of human origin using this antiserum; a similar band was detected in HepG2 cells by Dawson et al (1985), who suggested that it might be due to an abnormally glycosylated PB_{2c} protein. The exact relationship of this protein to PB_{2c} remains to be elucidated: if it is indeed an abnormally processed PB_{2c} protein it might have a significant effect on the patient's pulmonary drug metabolising capacity. It was intriguing that neither the lung cell lines used, nor the human lung samples examined, expressed high levels of isozyme PB_{3a}, although this isozyme is expressed at high levels in the lungs of other mammalian species (Wolf et al (1980)). Instead of PB_{3a}, the human lung samples studied appeared to express detectable levels of the related isozyme PB_1, as mentioned above. It would be of interest to examine further the relationship between these families of P-450s in human lung. Low, but detectable, levels of a protein immunochemically related to rat MC_{1b} were detected in the human lung and colon samples. As in the tumour-derived cell lines, the protein detected had slightly faster mobility than purified rat MC_{1b}; the possible relationship of this protein to those detected in human liver by Western blot analysis is discussed above. There was no evidence for any induction of this P-450 in either lung or colon samples, which was surprising since cigarette smoking might have been expected to induce expression of MC_{1b} in these lung cancer patients.

No consistent differences in P-450 expression between normal and tumour lung and colon samples were detected by Western blot anaysis. In some cases the levels of proteins immunochemically related to P-450s were elevated in the tumour sample relative to the corresponding normal sample (for example in lung tumour No. 6 and colon tumour No. 6); in these samples labelling with all four antisera was increased in the tumour. However, in the other samples there was relatively little difference in P-450 expression between normal and tumour material. The number of samples studied was not sufficiently large to draw conclusions about any relationship between tumour type and P-450 expression in the lung and colon. The lung sample which showed the most intense labelling with anti-P-450 antisera, No. 6, was from a male patient bearing a poorly differentiated squamous cell carcinoma; however, the group included several other patients with tumours of this type which did not have such high levels of P-450 expression. The colon sample having the highest levels of proteins immunochemically related to P-450s, No. 6, came from a 70 year old female patient. Only one other patient in the colon cancer group was female; further studies would be required in order to discover whether there is a sex difference in P-450 expression in the human colon.

Both human solid tissue samples and cell lines contained measurable levels of alkoxyresorufin metabolising enzymes. The activities detected were somewhat lower in the human tumour material than in the cell lines, possibly because the samples had been subjected to a number of freeze-thaw cycles in the course of microsome preparation and Western blot analysis whereas the cell samples were assayed on the first or second freeze-thaw cycle. The observed suppression of EROD activity in lung tumours relative to normal lung parenchyma agrees with the data of de Flora et al (1987), who showed that AHH activity was significantly lower in lung tumours than in normal lung parenchyma. It was interesting that no correlation between AHH levels and the number of cigarettes smoked was found in that study. A more recent study by the same group found that although there was no clear relationship between lung AHH activity and number of cigarettes smoked, there did seem to be a relationship between the time elapsing after ceasing smoking and AHH activity, lungs of recent smokers having elevated AHH activity compared with those who had ceased smoking more than thirty days before resection (Petruzzelli et al (1988)). The expression of P-450s in the series of colon samples studied shows that human colon mucosa and tumours also have the capacity to activate drugs and carcinogens by P-450-dependent pathways. Few previous studies on P-450-dependent activities in

human colon exist, although pioneering work by Wattenberg et al (1962) showed that AHH activity could be measured in human colon. Further studies are required in order to assess the significance of the present finding that BROD activity was significantly reduced in colon tumours relative to normal mucosa.

The results of this project, which demonstrate the existence of immunochemically detectable P-450 expression as well as the capacity to metabolise model substrates in human lung and colon, are complementary to the detailed studies on carcinogen metabolism performed by Harris using organ explants in short-term culture. Using this method it was shown that explants of human bronchus and colon could activate carcinogens such as B(a)P forming the same B(a)P-DNA adducts as those observed in experimental animals (Harris et al (1984)). Adduct formation by human bronchus was dependent on time, temperature and substrate concentration: the fact that this process was inhibited by 7,8-benzoflavone indicated that P-450 was involved. Interindividual differences of at least an order of magnitude in carcinogen-activating capacity were observed between human bronchi. In these studies a relationship between carcinogen activation and tumour type was observed; there was no significant difference in carcinogen-activating capacity between non-cancer samples and samples from patients bearing well-differentiated adenocarcinomas, but patients bearing primary epidermoid differentiated tumours had elevated B(a)P metabolising capacity. A family history of lung cancer was associated with increased B(a)P activation by lung explants.

Further studies on the lung tumour cell line NCI H322 showed that induction was reproducible and could be detected as an increase in the level of mRNA as well as MC_{1b} protein. The optimum concentration of BA for induction in this cell line was 3 - 5μg/ml (13 - 22μM). Concentrations of BA in the range 12 - 25μM have been shown to be optimal for induction experiments both in primary culture systems (Gielen and Nebert (1971a,b)) and in cell lines such as Hepa-1 and HepG2 (Hankinson (1979), Miller and Whitlock (1981), Grant et al (1988)). The growth medium giving the greatest ratio of induced versus basal activity was RPMI; this medium was selected for further experiments on P-450 induction in NCI H322 cells. Of the three media tested, RPMI, William's E and Minimal Essential Medium, the medium in which NCI H322 cells had the highest basal EROD activity was William's E. This medium has previously been shown to be able to support the

maintenance of P-450 expression in primary hepatocytes (Grant et al (1985), Steward et al (1985)), and HepG2 cells grown in this medium have relatively high basal EROD activity compared with those grown in DMEM (Doostdar et al (1988)).

In order to facilitate the complete characterisation of P-450 expression in NCI H322 cells, the most important requirement is to identify unequivocally the isozyme induced by BA in these cells. Human MC-inducible P-450 consists of two isozymes (MC_{1a} and MC_{1b}) both of which may be inducible by PAHs. The Northern blot, Western blot and enzymic data discussed above were consistent with the induced enzyme in NCI H322 cells being either of these forms. In order to identify the isozyme more precisely, two approaches are available. The first is to carry out antibody inhibition experiments using a range of antisera, or preferably monoclonal antibodies, raised against the two isozymes in order to discover which of these inhibits the observed activity. The second approach is to synthesise oligonucleotide probes complementary to the genes for the two proteins in a region in which their sequence differs, such as the 3' untranslated region, and to use these as probes on Northern blots of BA-induced NCI H322 cellular RNA. Similar experiments are necessary to identify the isozyme(s) induced by Aroclor 1254 in this cell line. Another aspect of P-450 induction in NCI H322 cells which has not yet been considered is the time course of induction. All the experiments described above, the cell were exposed to inducing agents for 24 hours (Aroclor 1254, BA) or 48 hours (PB, Dex). For complete characterisation of induction in these cells a detailed time course of the response to inducing agents is required in order to identify the optimal conditions for P-450 induction. Few human cell lines have been shown to contain a functional Ah receptor; in view of the observation that P-450 induction by the Ah ligand BA occurs in this cell line it would also be of interest to search for the Ah receptor in this cell line and to examine the properties of the receptor if found.

In this chapter it has been established that a number of human tumour cell lines express P-450s which can be induced by BA. The cell lines NCI H322, of human lung adenocarcinoma origin, and HepG2, of human hepatoblastoma origin, were chosen for further study on the basis of their inducibility and ease of growth. Their use in studies of the metabolic activation of B(a)P and cyclophosphamide are described in Chapter 4, and the use of NCI H322 cells to study the effects of inflammatory mediators on P-450-dependent enzyme activity is described in Chapter 5.

Chapter 4.

Effects of P-450-inducing agents on susceptibility to cytotoxins: Use of the MTT assay.

4.1. Aims

The aims of this part of the project were:

(i) To characterise the behaviour of HepG2 and NCI H322 cells in the MTT assay and to use this assay to determine the toxicity of P-450-inducing agents.

(ii) To discover whether pretreatment of HepG2 and NCI H322 cells with a non-toxic dose of an inducing agent will alter their susceptibility to carcinogens or anticancer drugs which are known to be activated by the enzyme induced.

4.2. Metabolic activation of drugs and carcinogens.

4.2.1. In vitro systems used to study metabolic activation.

Many carcinogens and toxic drugs are themselves chemically inert but are activated by various routes within cells to form highly toxic metabolites. This process is called "metabolic activation" and is frequently part of the normal metabolic pathway. The observed toxicity of such chemicals depends on the balance between activation and detoxification, which is often dependent on the cell's spectrum of P-450s and detoxifying enzymes; other enzyme systems such as peroxidases and prostaglandin synthetase may also be involved (Haim et al (1986)). The phenomenon of metabolic activation of chemicals may be deleterious to the organism, as in the activation of carcinogens to form intermediates which bind to DNA, or beneficial, for example in the case of activation of an anticancer drug within the tumour cell. A wide variety of in vitro systems are used to assess the metabolic activation of chemicals to mutagenic or cytotoxic metabolites using either genetic end-points such as mutation and sister chromatid exchange, or non-genetic ones such as cytotoxicity (Dunkel (1983)).

Among the most popular system for assessing the formation of mutagenic metabolites is the Ames test, which uses a series of strains of Salmonella typhimurium to detect mutations converting the bacteria from the His^- to the His^+ phenotype. Originally, only compounds which did not require metabolic activation were detected by this test (Ames et al (1973a)), but the test was modified by the inclusion of a hepatic 9000g supernatant (S9) fraction, enabling the detection of mutagens requiring metabolic activation. Using this test, a strong correlation between in vitro mutagenicity and carcinogenicity was reported (Ames et al (1973b, 1975), McCann and Ames (1976)). It was possible to assess the susceptibility of a compound to metabolic activation by comparing its mutagenicity in the presence and absence of S9. As well as testing compounds for mutagenicity, the Ames test has been used to elucidate pathways of metabolic activation by antibody inhibition experiments (Kawajiri et al (1983)) and by incorporating purified P-450s or S9 from various organs as an activation system (McManus et al (1984), Robertson et al (1981)). The major disadvantage of the Ames test lies in extrapolating from in vitro activation by S9 to activation in the living cell, which may be quite different due to the presence of enzymes which are absent or non-functional in S9 and to compartmentalisation within the cell. One approach to this problem has been the use of cultured cells such as primary hepatocytes or HepG2 cells as an activating system (Zhuo et al (1986)).

Mutagenicity may also be measured in mammalian cells, including Chinese hamster ovary (CHO) cells, hamster lung V79 cells and mouse L5178Y cells. The commonly used markers are those involving mutation of the hypoxanthine guanine phospho-ribosyl transferase and thymidine kinase genes. Resistance to the Na^+/K^+ ATPase inhibitor ouabain may also be measured. In these assays the target cells are usually unable to perform metabolic activation, and a second cell type is included to carry out activation in "cell-mediated assays". Lethally irradiated hamster embryo cells, which retain reasonably high levels of P-450 activity, are a popular activation system (Huberman and Sachs (1974)); primary rat hepatocytes have also been used (San and Williams (1977)). The HepG2 cell line has also become popular as an activating system and has the advantage that, being of human origin, it is expected to activate compounds via the same pathways as human liver. Lethally irradiated HepG2 cells are able to activate a number of PAHs, including B(a)P, to products mutagenic towards V79 cells (Diamond et al (1980, 1984), DiGiovanni et al (1984)). Sister chromatid exchange is another end-point used to assess mutagenicity (Wolff

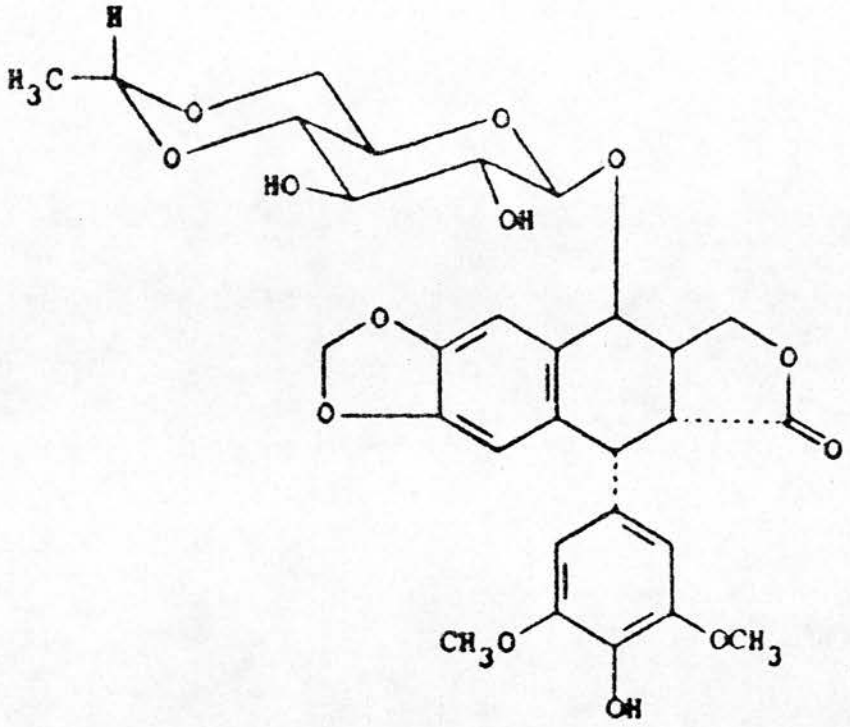
(1983)). This assay involves visualisation of sister chromatid exchanges in cells treated so that the chromatids may be distinguised physically or chemically. Chemicals which require no metabolic activation in order to cause sister chromatid exchanges may be readily detected; compounds requiring metabolic activation may be detected by adding an activating system. In some cases, induction of sister chromatid exchanges in the activating cell itself are measured. Experiments of this kind showed that the drugs diethylstilbestrol and cyclophosphamide induce sister chromatid exchange in HepG2 and H4-II-E cells, but not in V79 or MGH fibroblasts, implying that metabolic activation in the two liver lines is important in induction of sister chromatid exchange (Buenaventura et al (1984), Dearfield et al (1983, 1986)).

Production of intermediates which are cytotoxic, either to a target cell or to the activating cell itself, may also be used to asses metabolic activation. Lethally irradiated mouse fibroblasts are able to activate dimethylbenzanthracene to products which are cytotoxic to neoplastic cells (Mittleman et al (1972)), and lethally irradiated HepG2 cells activate B(a)P to intermediates which kill V79 cells (Limbosch (1983)). The classic example of the activation of B(a)P to products resulting in toxicity towards the activating cell itself is the system used to select B(a)P resistant mutants of the mouse liver cell line Hepa-1, discussed previously (Hankinson (1979)). In view of the results discussed above, which show that HepG2 cells are also able to activate B(a)P to both cytotoxic and mutagenic products, the question arises whether, and to what degree, modulation of the P-450 level of this cell line alters its susceptibility to the cytotoxic effects of B(a)P.

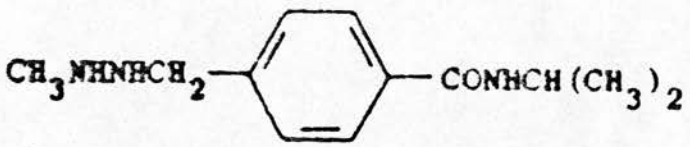
4.2.2. P-450 mediated metabolic activation.

Metabolic activation of drugs and carcinogens by specific P-450s is important since it often results in the formation of highly reactive intermediates such as epoxides (Jollow and Smith (1977)). Various types of compounds undergo P-450-dependent metabolic activation, including organic solvents such as benzene, aromatic amines such as 2-acetylaminofluorene, and drugs such as acetaminophen (Mitchell et al (1973 a,b), Jollow et al (1973), Potter et al (1973), Wolff (1983) Kawajiri et al (1983), McManus et al (1984)). In this section the importance of P-450-dependent metabolic activation will be illustrated by discussing two anticancer drugs, procarbazine and VP-16 (etoposide); the two compounds used in this study, B(a)P and cyclophosphamide will also be introduced. The structures of these compounds are shown in Figure 4.1.

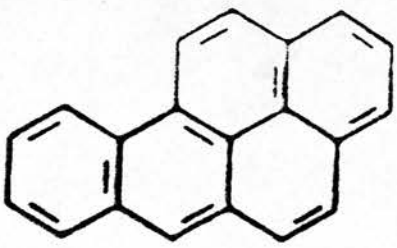
Figure 4.1.
Structures of four compounds which undergo P-450-mediated metabolic activation.



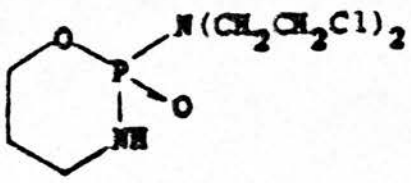
(a) VP-16



(b) Procarbazine



(c) Benzo(a)pyrene

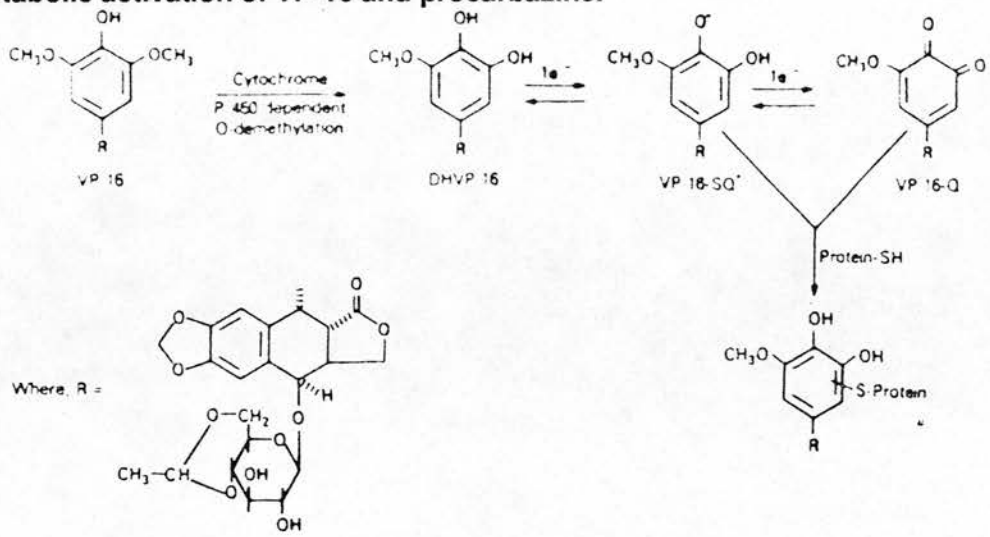


(d) Cyclophosphamide

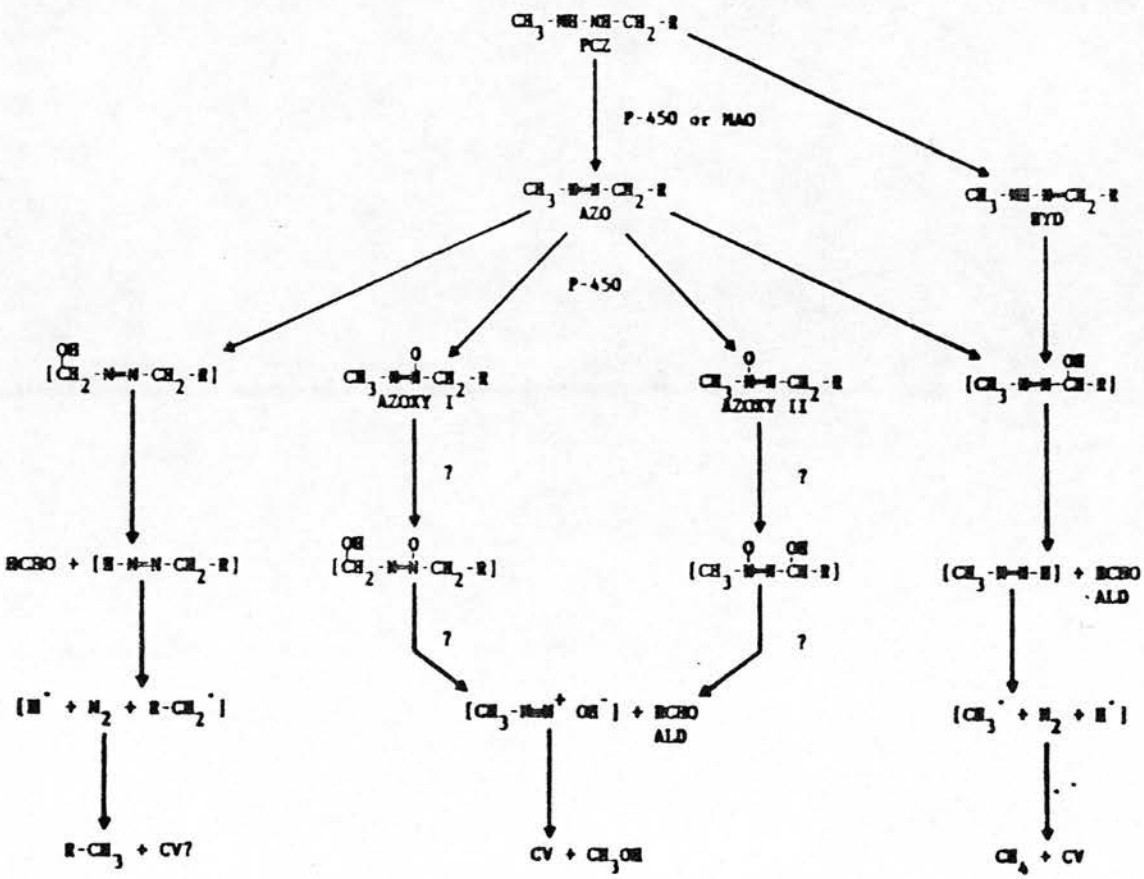
The semisynthetic podophyllotoxin derivative VP-16 (4'-dimethyl-9-[4,6-O-ethylidine-D-glucopyranoside]) is used to treat several types of tumour, including small-cell lung carcinoma, testicular cancer, lymphoma and Hodgkins disease (O'Dwyer et al (1985)). Its metabolism follows two major pathways; opening of the lactone ring by esterases results in detoxification whereas O-demethylation appears to be an activation route (Van Maanen et al (1982), Sinha and Myers (1984)) (Figure 4.2). The products of VP-16 O-demethylation were shown to form DNA-adducts (Sinha and Myers (1984)), but could also remove toxic free radicals produced as a result of treatment with drugs such as daunomycin (Sinha et al (1985)). VP-16 was O-demethylated by peroxidase, prostaglandin synthetase and P-450-dependent pathways (Haim et al (1986,1987)). In studies aimed at identifying the P-450 involved in O-demethylation of VP-16 PB_{3a} catalysed the reaction by monooxygenase and peroxidase pathways (in the presence of NADPH and cumene hydroperoxide, respectively). However, treatment of rats with 3-MC quantitatively induced metabolism of VP-16 to a catechol product which is cytotoxic to CHO and H35 cells and binds efficiently to calf thymus DNA (Van Maanen et al (1987)). This suggested, but did not prove, that MC_{1b} was involved in the O-demethylation of VP-16 in rat liver microsomes; further studies should help to clarify the pathways of VP-16 activation.

Procarbazine (N-isopropyl-a-(2-methylhydrazine)-p-toluamide), which was originally synthesised as a prospective monoamine oxidase inhibitor and is now used in the treatment of Hodgkin's lymphoma, is particularly interesting because it undergoes the series of P-450-mediated metabolic activation steps shown in Figure 4.2 (Prough and Tweedie (1987)). The metabolism of procarbazine has been extensively studied since the 1960s, when it was shown to undergo enzymatic or spontaneous oxidation followed by N-demethylation resulting in the release of formaldehyde (Raaflaub and Swartz (1965), Schwarz et al (1967)). The actual substrate for N-demethylation was azoprocarbazine, produced spontaneously in the presence of oxygen or after microsomal metabolism by P-450 or monoamine oxidase (Wittkop et al (1969)). The P-450-mediated formation of azoprocarbazine was induced by PB and inhibited by metyrapone (Dunn et al (1979)). Further microsomal metabolism of azoprocarbazine produced two azoxyprocarbazine derivatives, methyl- and benzyl-azoxyprocarbazine. It was proposed that the methylazoxyprocarbazine derivative was responsible for the cytotoxicity of

Figure 4.2.
Metabolic activation of VP-16 and procarbazine.



(a) Structures of VP-16, 3',4'-dihydro-VP-16 (DHVP-16) and the o-quinone derivative of VP-16 (VP-16-Q) and proposed cytochrome P-450-dependent metabolism of VP-16 resulting in the formation of VP-16-Q and VP-16 semiquinone free radical (VP-16-SQ·)(From Haim et al (1987)).



(b) The metabolic pathway of procarbazine activation.
PCZ = procarbazine; AZO = azoprocarbazine; AZOXY I = benzylazoxyprocarbazine; AZOXY II = methylazoxyprocarbazine; MAO = monoamine oxidase; CV = covalent binding; R = $-C_6H_4-CONHCH(CH_3)_2$. (From Prough and Tweedie (1987)).

procarbazine, possibly acting via formation of a methyldiazonium ion (Weibkin and Prough (1980)). Both MC_{1b} and PB_1 were able to metabolise azoprocarbazine to form methylazoxyprocarbazine, MC_{1b} accounting for 80% of this activity in rats treated with β-naphthoflavone whereas PB_1 was more important in uninduced animals (Prough et al (1984)). Procarbazine is therefore very interesting from the point of view of metabolic activation since a number of P-450s are involved; further studies on the roles of specific P-450s in this process are merited.

Many model compounds are suitable for metabolic activation studies. Since the present study was to be carried out using HepG2 cells it was decided to adopt a compound whose activation has been extensively studied in cultured cells as well as in vivo, namely B(a)P. As a control, cyclophosphamide was chosen to represent a compound which was not expected to be activated by MC-inducible P-450s.

4.2.3. Metabolic activation of benzo(a)pyrene.

B(a)P is an important carcinogen because it is very potent and is found in measurable quantities in the environment as a result of pollution . Levels as high as 40μg/m^3 have been reported in heavily polluted air (Doll and Peto (1981)). Metabolic activation of B(a)P was thoroughly reviewed by Gelboin (1980) and Conney (1982); the majority of this introduction to B(a)P activation will be based on these two sources. It was shown many years ago that there was a correlation between the levels of AHH in cells and their susceptibility to the toxic effects of B(a)P (Gelboin et al (1969)). Studies by Holder et al (1974) showed that the metabolism of B(a)P by liver microsomes was enhanced by both PB and 3-MC treatment, but that the two inducing agents resulted in significant differences in the pattern of metabolites detected by high pressure liquid chromatography. The major stable products detected were phenols, quinones, and diols. Most of the phenol and quinone metabolites were relatively non-mutagenic (Wislocki et al (1976), although the diols and quinones were important in the cytotoxicity of B(a)P since they participated in redox cycles generating highly toxic oxygen free radicals (Lorentzen and Ts'o (1977)).

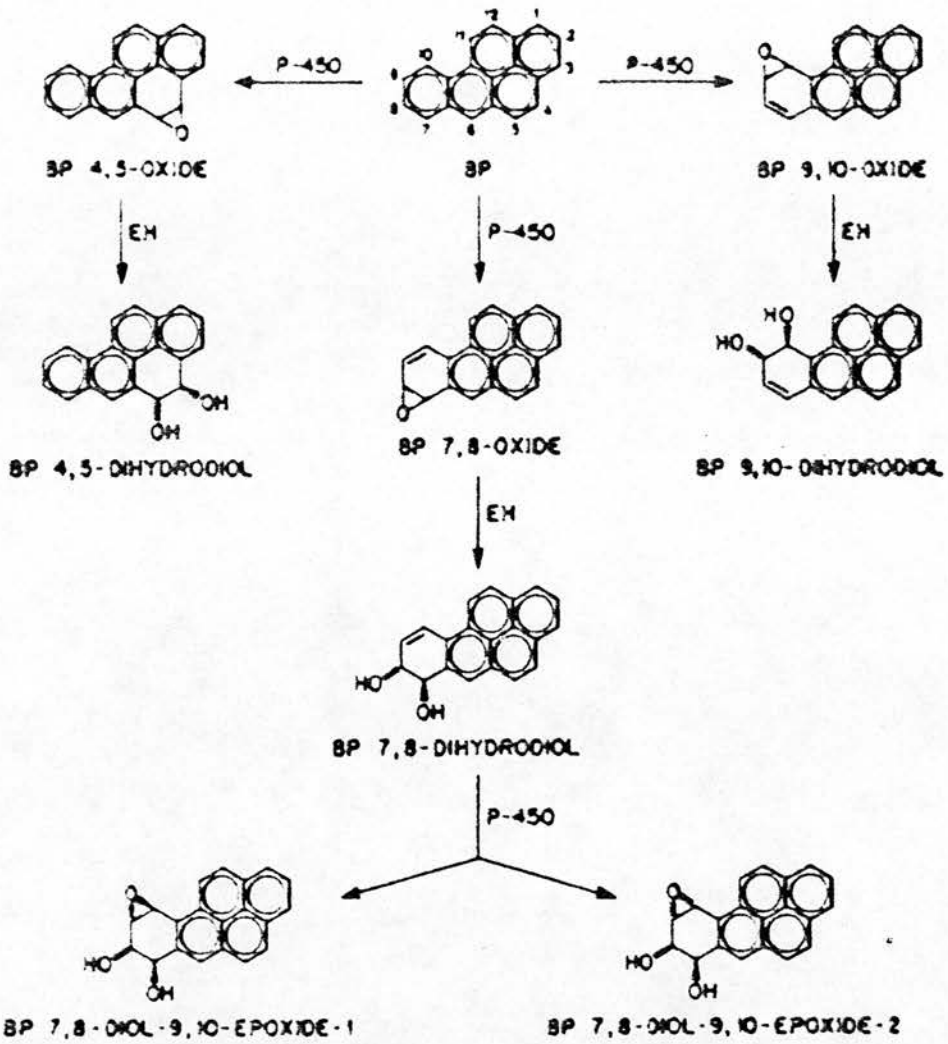
It was soon shown that a reconstituted system containing purified MC_{1b} metabolised B(a)P to a mixture of phenols and quinones, but not to dihydrodiols unless epoxide hydrolase was present (Wood et al (1976a)). On addition of epoxide hydrolase (EH),

the 4,5-, 7,8- and 9,10- trans-dihydrodiols of B(a)P were also detectable by high pressure liquid chromatography. It was inferred that the primary metabolites of B(a)P in the reconstituted system were the (4,5), (7,8) and (9,10)-epoxides, although only the 4,5-epoxide was stable enough to detect. Dihydrodiols formed by the action of EH on the epoxide primary metabolites of B(a)P were themselves subject to further P-450-mediated epoxidation forming diol epoxides which proved to be the major mutagenic products of B(a)P activation. The most active of these diol epoxides was the 7,8-diol-9,10-epoxide; the pathway proposed as the major route of mutagenic activation is shown below (A more detailed scheme for the metabolic activation of B(a)P is shown in Figure 4.3a).

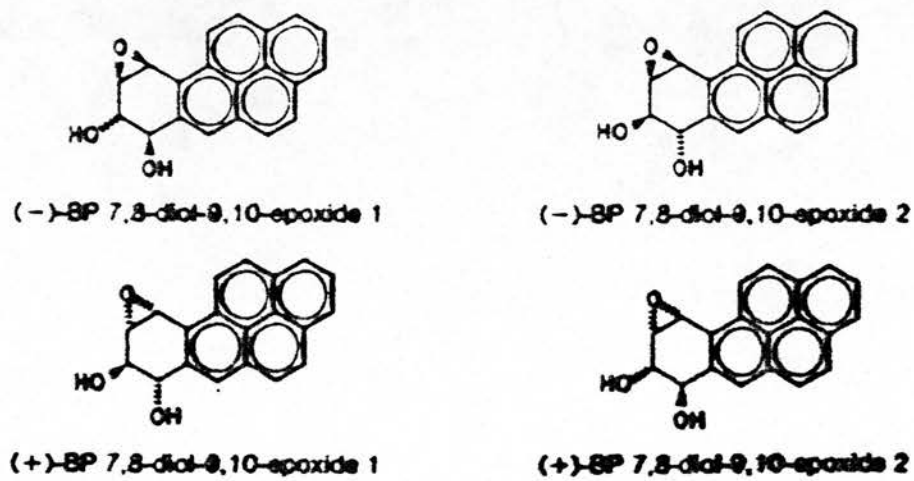
	P-450	EH	P-450
B(a)P	-> B(a)P-7,8-oxide	-> B(a)P-7,8-diol	-> B(a)P-7,8-diol-9,10-epoxide.

The sequence of epoxidation by P-450s followed by hydration by EH led to the formation of trans-B(a)P-7,8-dihydrodiol. Chromatographic comparison of the DNA adducts formed in hamster embryonic cells suggested that the major DNA binding metabolite of B(a)P was the 7,8-diol-9,10-epoxide which exists in two racemic forms, diol epoxides I and II (Figure 4.3b). Both were predicted to be highly mutagenic, probably acting via the formation of a C-10 carbonium ion which could attack cellular nucleophiles. Metabolites of B(a)P were studied in a number of systems, including the S9-mediated Ames test, the hamster embryo cell-mediated system, a direct assay using V79 cells and a human kidney carcinoma cell mediated assay (Malaveille et al (1975), Newbold and Brookes (1976), Wislocki et al (1976b), Huberman et al (1976), Aust et al (1980)). The data confirmed that the 7,8-diol-9,10-epoxides were the most mutagenic metabolites of B(a)P, although B(a)P-4,5-oxide was also strongly mutagenic. However, there was considerable debate as to which of the diol epoxides was the most important mutagenic form in vivo; the relative activities of the two forms depended on the mutagenicity test used. One reason for the potency of the diol epoxides as mutagens may be their resistance to detoxification by EH; other potentially mutagenic metabolites were hydrolysed to form weakly mutagenic phenols by EH (Wood et al (1976b). The major site of attack by B(a)P diol epoxides in DNA is the N^2-atom of guanine. Adducts at this site cause secondary structure effects which make DNA polymerase hesitate leading to errors in replication. Diol epoxides can also attack adenosine residues resulting in local unwinding of the double helix and errors in replication.

Figure 4.3.
Metabolic activation of benzo(a)pyrene.



(a) Metabolism of B(a)P to arene oxides, dihydrodiols, and the diastereomeric B(a)P-7,8-diol-9,10-epoxides. (From Conney (1982)).



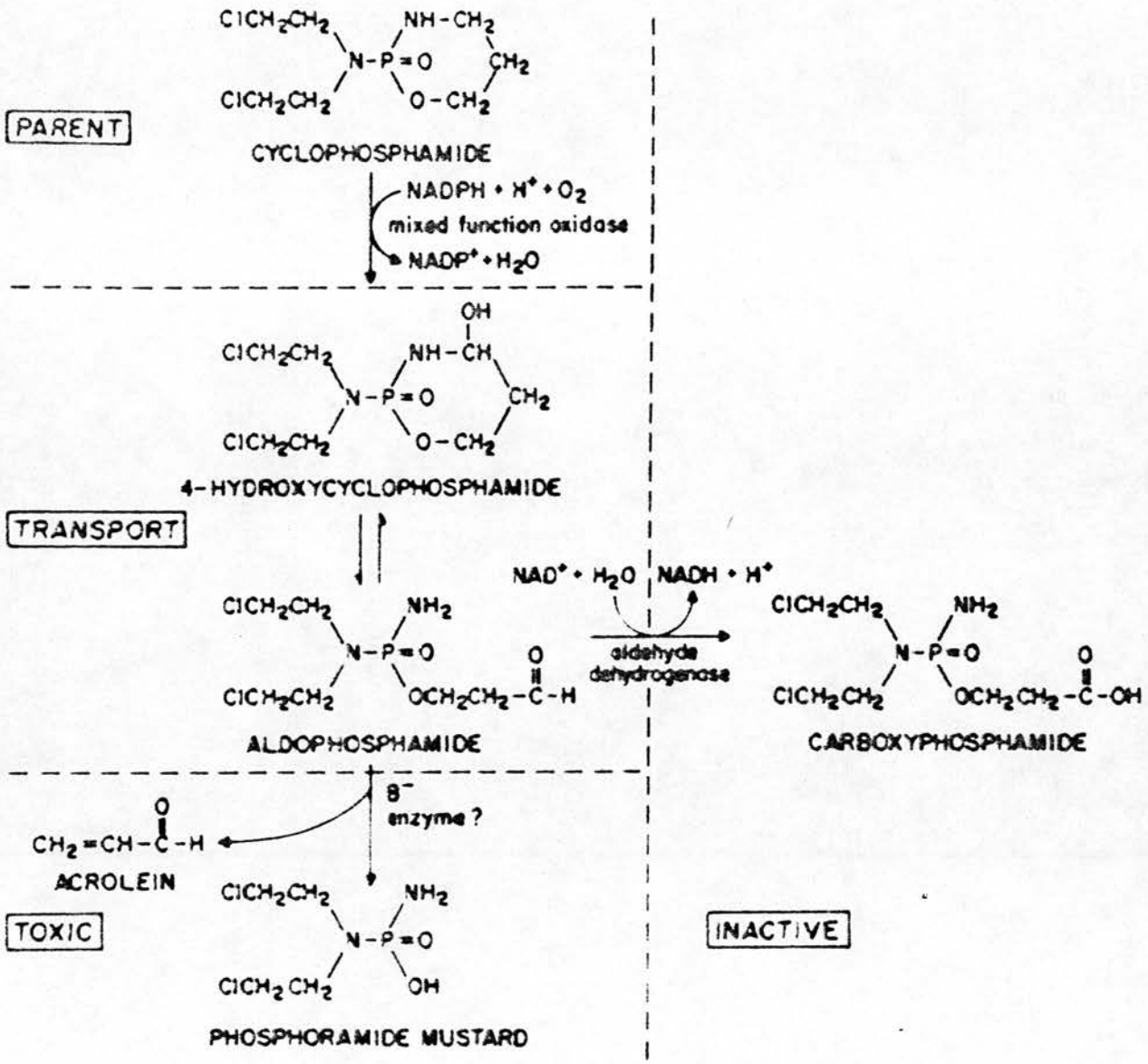
(b) Structures of the diastereomeric B(a)P-7,8-diol-9,10-epoxides, probable mutagenic metabolites of B(a)P. (From Conney (1982)).

Human liver and lung-derived cell lines are capable of activating B(a)P to form mutagenic diol epoxides (Aust et al (1980), Limbosch (1983), Diamond et al (1980,1984), DiGiovanni et al (1984)). The hypothesis tested in the present study is that, since MC_{1b} is induced in HepG2 and NCI H322 cells treated with BA, the induced cells are expected to activate B(a)P to toxic intermediates to a greater extent, and thus suffer more B(a)P-induced cytotoxicity, than control cells.

4.2.4. Metabolic activation of cyclophosphamide.

Cyclophosphamide (2-chloroethylamido-oxazophosphorine), used to treat a wide variety of tumours and autoimmune diseases, was designed to undergo metabolic activation to a cytotoxic form within tumour cells (Sladek (1987)). Early studies, however, revealed that the liver was its major site of activation: it appeared to be metabolised by a P-450-dependent mechanism, possibly by the enzyme which metabolised ethylmorphine (Sladek (1971)). Microsomes from rats treated with PB were shown by kinetic analysis, alkylating metabolite formation and mutagenicity to be highly efficient at metabolising cyclophosphamide; the reaction was inhibited by SKF 525A, suggesting that isozyme PB_{3a} might be responsible (Field et al (1972), Sladek (1972a,b), Hales and Jain (1980)). Further studies showed that activation of cyclophosphamide proceeded via the tautomers 4-hydroxycyclophosphamide and aldophosphamide (Sladek (1973)), which underwent spontaneous β-elimination releasing phosphoramide mustard and acrolein (Conners et al (1974) (Figure 4.4). The proposed mode of action of cyclophosphamide involved formation of 4-hydroxy-cyclophosphamide, followed on entering the target cell by spontaneous decomposition releasing phosphoramide mustard and acrolein. Phosphoramide mustard was shown to be a metabolite of cyclophosphamide in humans (Fenselau et al (1975)). Use of a reconstituted monooxygenase system containing PB_{3a} and cyclophosphamide labelled in different regions of the molecule confirmed that isozyme PB_{3a} was able to form phosphoramide mustard and acrolein from the drug (Colvin et al (1976)). In covalent binding assays using hepatic microsomes from PB-treated rats phosphoramide mustard was the major DNA-binding metabolite; acrolein bound more readily to proteins including the P-450 molecule itself (Marinello et al (1985)). Binding of phosphoramide mustard to DNA was thought to proceed via formation of a highly electrophilic aziridium ion. Phosphoramide mustard appeared to exert the majority of the mutagenic effects of cyclophosphamide by binding to DNA, while acrolein may have been responsible for some of the cytotoxic effects observed.

Figure 4.4.
Metabolic activation of cyclophosphamide.



Metabolism of cyclophosphamide: an abridged scheme showing only the currently recognised biologically important reactions and metabolites. (From Sladek (1987)).

Recent studies showed that HepG2 cells were able to activate cyclophosphamide by a mechanism which is inhibited by SKF 525A (Dearfield et al (1983, 1986)). Cytotoxicity due to cyclophosphamide was not examined in that study. In the present study, the toxicity of cyclophosphamide towards control and BA-treated HepG2 and NCI H322 cells was examined in order to rule out additive cytotoxic effects or other effects of the inducing agent on the cells. The prediction made was that, since the only isozyme induced in this system was thought to be MC_{1b}, and the major isozyme involved in metabolic activation of cyclophosphamide is thought to be PB_{3a}, there would be no difference between control and BA-treated cells in susceptibility to cyclophosphamide toxicity.

4.3. Cytotoxicity of P-450 inducing agents.

4.3.1. Behaviour of HepG2 and NCI H322 cells in the MTT assay.

Carmichael et al (1987a,b) placed great emphasis on characterising the behaviour of each cell line to be used in the MTT assay. The following experiments were carried out in order to fulfil this requirement for HepG2 and NCI H322 cells.

(a) Dependence of absorbance after MTT treatment on cell number: Use of the MTT assay to test cytotoxicity depends on assuming that the relationship between the OD of the formazan product obtained after MTT treatment and the number of cells present in a well is linear. In order to confirm this, known numbers of cells were plated out and incubated for 2 - 3 hours until they had adhered but not started to divide. MTT was added and the plates processed as described. It was found that the relationship between OD and cell number approximated to a straight line over the range 0 - 50,000 cells per well, but above this range the relationship was not linear (Figures 4.5 and 4.6). For this reason, the results of cytotoxicity experiments were always plotted as "percentage of control OD" rather than "percentage survival" because of the imperfect linearity of the OD versus cell number relationship, which meant that it was not possible to extrapolate directly from OD to cell survival. However, in discussion of the results the assumption that a 50% reduction in OD represents a 50% reduction in cell survival will be made.

(b) Relationship of number of cells plated out to final cell number and OD: In the MTT assay it is important that the control cells have not reached confluency when the plates are processed, but the OD obtained should be high enough to ensure that small variations between wells do not affect the results. In order to choose a seeding density

to fulfil these criteria, various numbers of cells were plated out in sextuplicate on 96-well plates and allowed to grow for 6 days, the normal duration of an MTT assay. Half of the wells were treated with MTT and their ODs measured; the number of cells in the other wells was counted using a haemocytometer. The relationship between the number of cells plated out and the final number per well was linear when low numbers of cells were plated out but levelled off when high cell numbers were used (Figures 4.7(a) and 4.8(a)). The result of measuring OD after MTT treatment was similar, but the final OD reached a plateau at lower initial cell numbers than did the final cell number (Figures 4.7(b) and 4.8(b)). It was decided to plate out 10,000 HepG2 cells per well and 2500 NCI H322 cells per well for cytotoxicity assays. The final ODs and cell numbers were also plotted against each other (Figures 4.7(c) and 4.8(c)); the relationship was approximately linear over the same range of cell numbers as in the previous experiment.

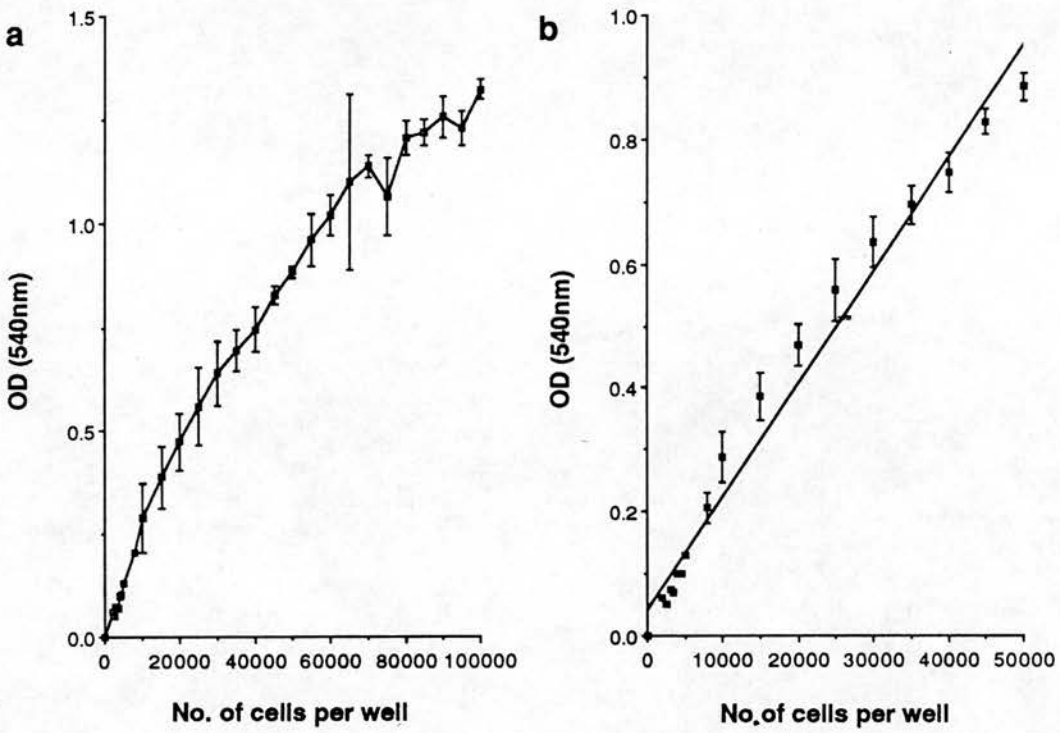
(c) Growth rate of cells on 96-well plates: In order to confirm that cells were in logarithmic growth throughout the MTT assay the growth of cells on 96-well plates was assessed using the MTT method (Figure 4.9). Six plates containing various numbers of cells per well were set up; each day for 6 days a plate was treated with MTT and processed. At low initial cell numbers, a lag phase occurred before the cells entered logarithmic growth. When high cell numbers were plated, the lag phase was brief and growth reached a plateau within the 6 days of the assay. This experiment showed that the cell number chosen for HepG2 cells (10,000 per well) was too high since the cells reached a plateau before the end of the assay; later experiments using this cell line were shortened to 4 days after drug treatment. The cell number chosen for NCI H322 cells (2500 per well) appeared to be appropriate since the cells were still in logarithmic growth at the end of the assay.

4.3.2. Cytotoxicity of P-450 inducing agents.

In order to ensure that the four inducing agents used in this project were not cytotoxic over the range of concentrations chosen for induction experiments the MTT assay was used to test their cytotoxicity. The results are shown in Figures 4.10 - 4.13 and summarised in Table 4.1. It should be noted that the percentage of control OD after MTT treatment has been plotted against the $\log_{10}$ of the inducing agent concentration. In each case the control used was solvent treatment alone, ie. 20µl serum-free medium in the case of PB or 0.25% DMSO in the other three cases.

Figure 4.5.

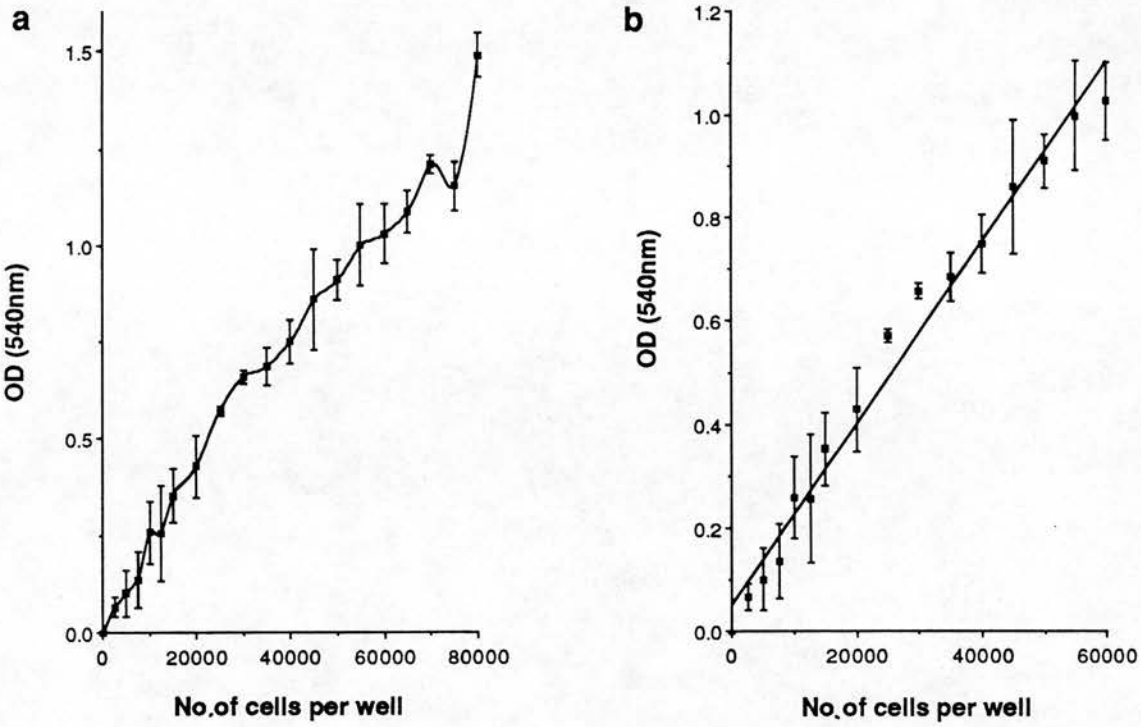
Relationship between number of HepG2 cells present and optical density obtained after MTT treatment.



Confluent HepG2 cells were harvested and the number of viable cells counted using a Neubauer haemocytometer. Various numbers of cells from 0 to 100,000 cells per well in a volume of 200µl of medium were seeded out in triplicate on 96-well plates and allowed to adhere for 2 - 3 hours. After this time, 50µl of MTT (2mg/ml) was added to each well and the plates incubated for a further 4 hours at 37°C. The supernatant was then removed by aspiration, the blue formazan crystals dissolved in 50µl of DMSO, and the OD (540nm) of each well read using a Biorad Model 2550 EIA reader. The graphs show:
(a) OD (540nm) vs cell number over the whole range tested.
(b) OD (540nm vs cell number over the range approximating best to linearity.

Figure 4.6.

Relationship between number of NCI H322 cells present and optical density obtained after MTT treatment.



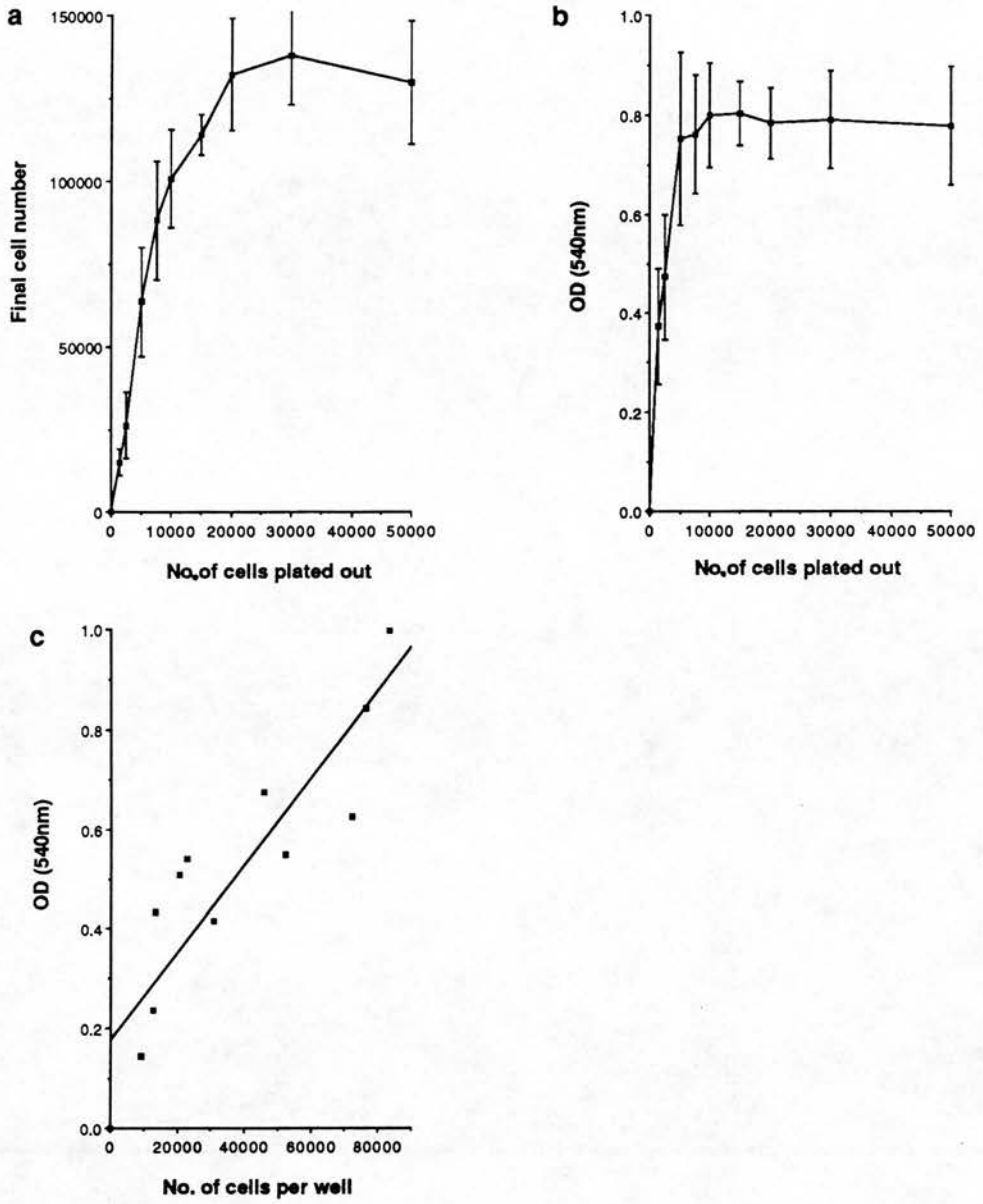
Confluent NCI H322 cells were harvested and the number of viable cells counted using a Neubauer haemocytometer. Various numbers of cells from 0 to 80,000 cells per well in a volume of 200µl of medium were seeded out in triplicate on 96-well plates and allowed to adhere for 2 - 3 hours. After this time, 50µl of MTT (2mg/ml) was added to each well and the plates incubated for a further 4 hours at 37°C. The supernatant was then removed by aspiration, the blue formazan crystals dissolved in 50µl of DMSO, and the OD (540nm) of each well read using a Biorad Model 2550 EIA reader. The graphs show:

(a) OD (540nm) vs cell number over the whole range tested.

(b) OD (540nm vs cell number over the range approximating best to linearity.

Figure 4.7.

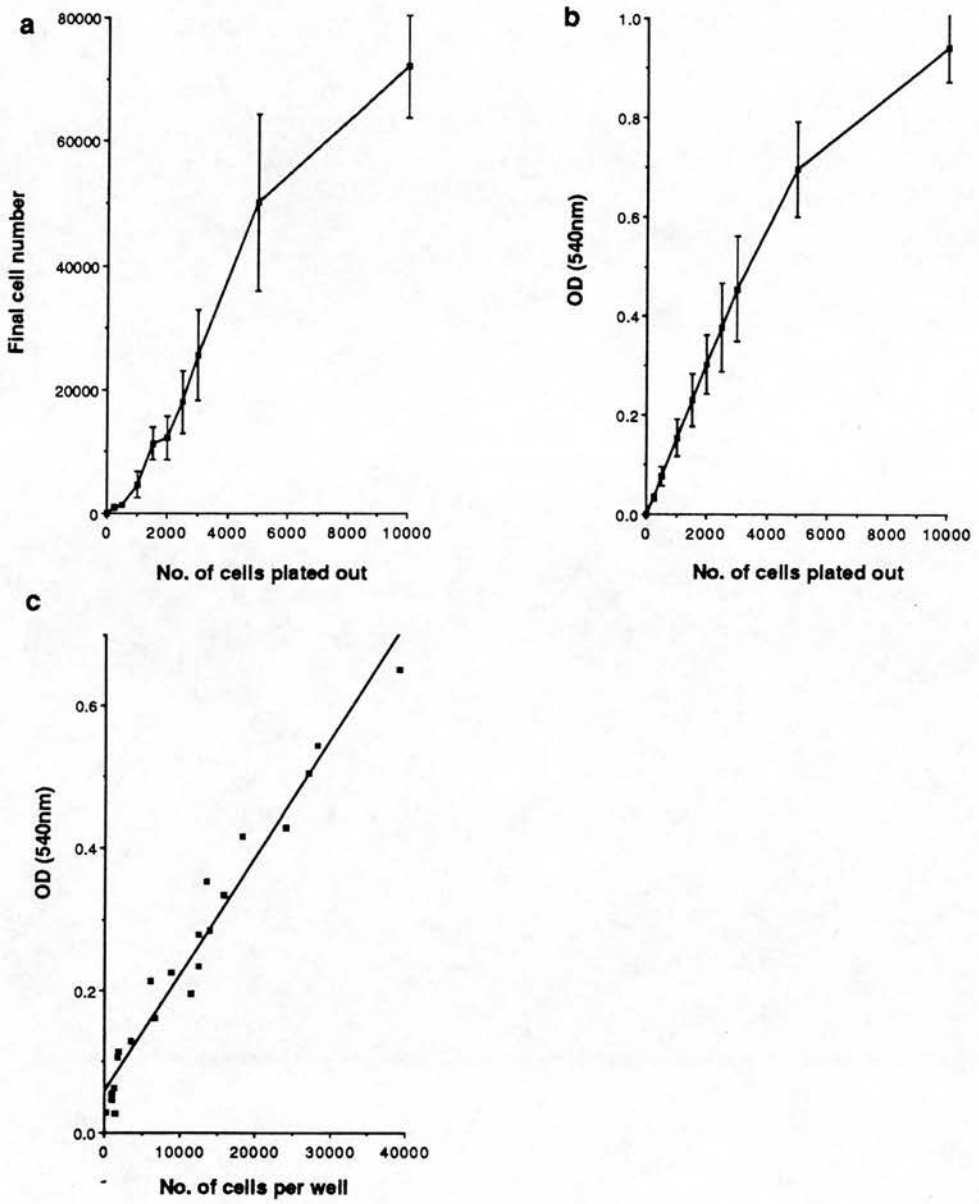
Relationship of number of HepG2 cells plated out to number of cells and optical density obtained after 6 days growth.



Confluent HepG2 cells were harvested and the number of viable cells counted using a Neubauer haemocytometer. Various numbers of cells from 0 to 50,000 cells per well in a volume of 200µl of medium were seeded out in sextuplicate on 96-well plates and allowed to grow for 6 days. After this time, 50µl of MTT (2mg/ml) was added to triplicate wells and the plates developed as described. The cells in the other three wells of each group were harvested by trypsinisation and counted.
(a) Number of cells after 6 days plotted against number of cells initially plated out.
(b) OD (540nm) plotted against number of cells plated out.
(c) OD (540nm) plotted against number of cells counted by trypsinisation (linear region of the curve).

Figure 4.8.

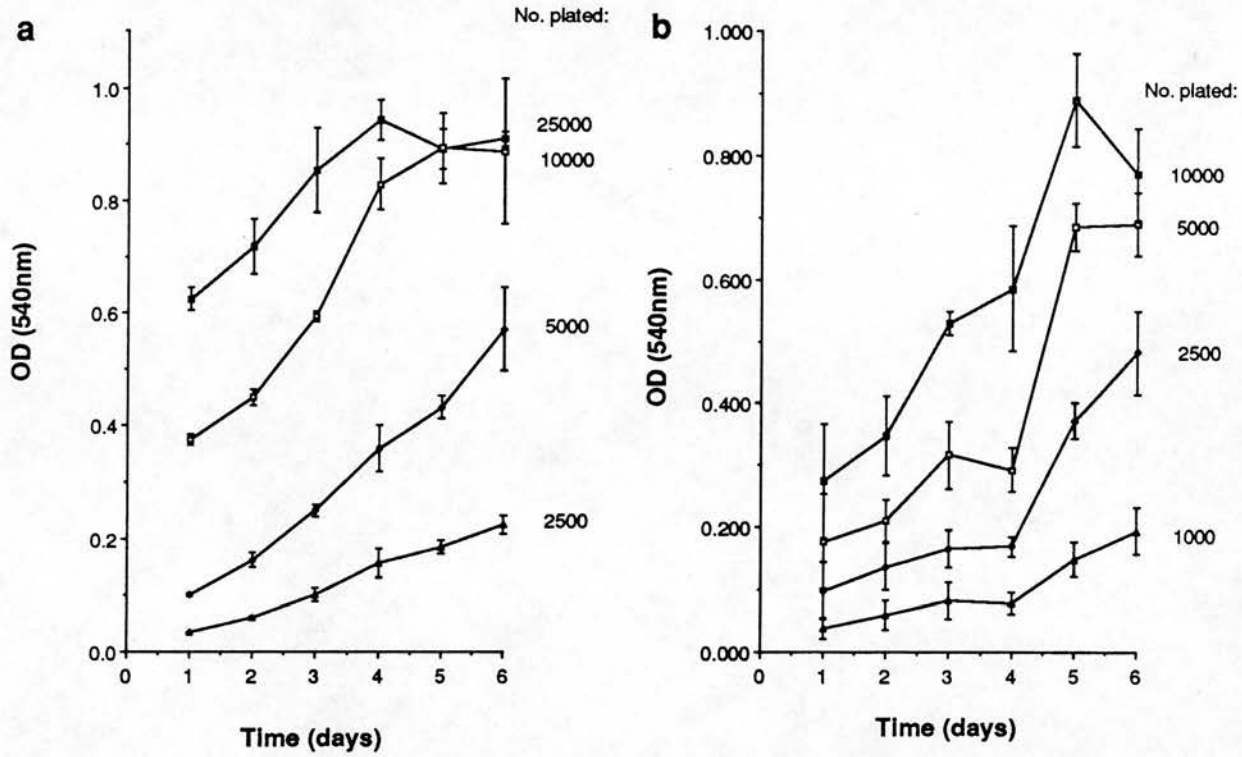
Relationship of number of NCI H322 cells plated out to number of cells and optical density obtained after 6 days growth.



Confluent NCI H322 cells were harvested and the number of viable cells counted using a Neubauer haemocytometer. Various numbers of cells from 0 to 10,000 cells per well in a volume of 200µl of medium were seeded out in sextuplicate on 96-well plates and allowed to grow for 6 days. After this time, 50µl of MTT (2mg/ml) was added to triplicate wells and the plates developed as described. The cells in the other three wells of each group were harvested by trypsinisation and counted.
(a) Number of cells after 6 days plotted against number of cells initially plated out.
(b) OD (540nm) plotted against number of cells plated out.
(c) OD (540nm) plotted against number of cells counted by trypsinisation (linear region of the curve).

Figure 4.9.

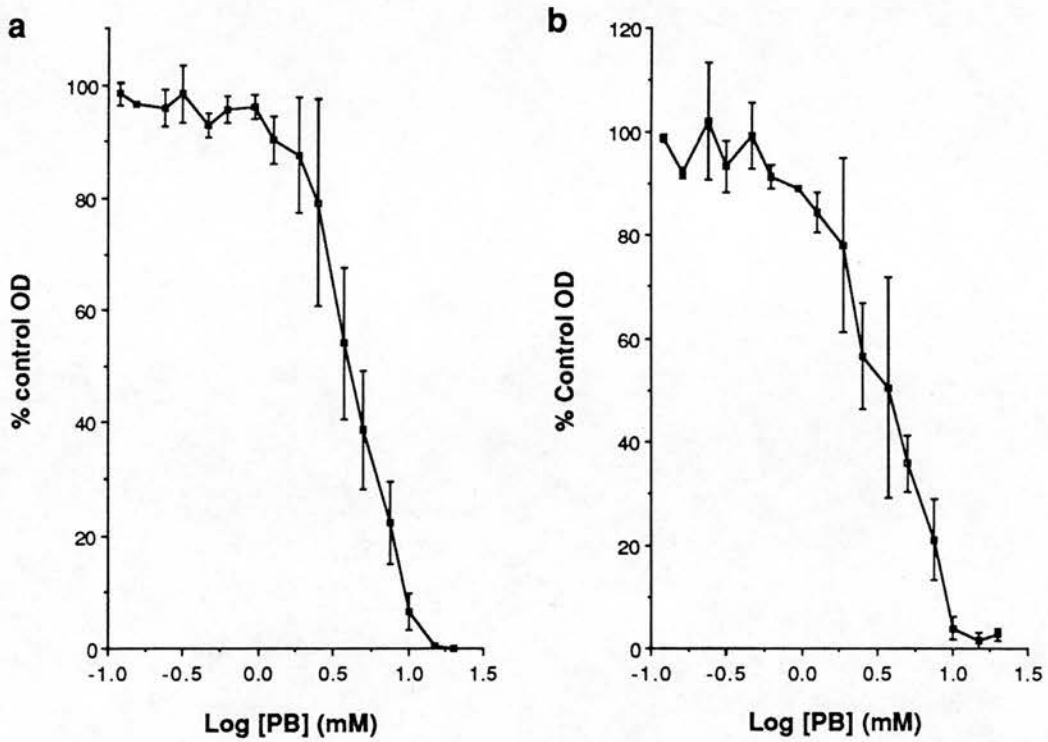
Growth rate of HepG2 and NCI H322 cells on 96-well plates.



Confluent HepG2 or NCI H322 cells were harvested and the number of viable cells counted using a Neubauer haemocytometer. Various numbers of cells from 0 to 25,000 cells per well in a volume of 200µl of medium were seeded out in sextuplicate on 96-well plates and allowed to grow for 6 days. On each day, 50µl of MTT (2mg/ml) was added to the cell on one plate and the plate developed as described.
(a) Increase in OD (540nm) of HepG2 cells after plating out various numbers of cells from 2500 - 25,000 per well.
(b) Increase in OD (540nm) of NCI H322 cells after plating out various numbers of cells from 1000 -10,000 per well.

Figure 4.10.

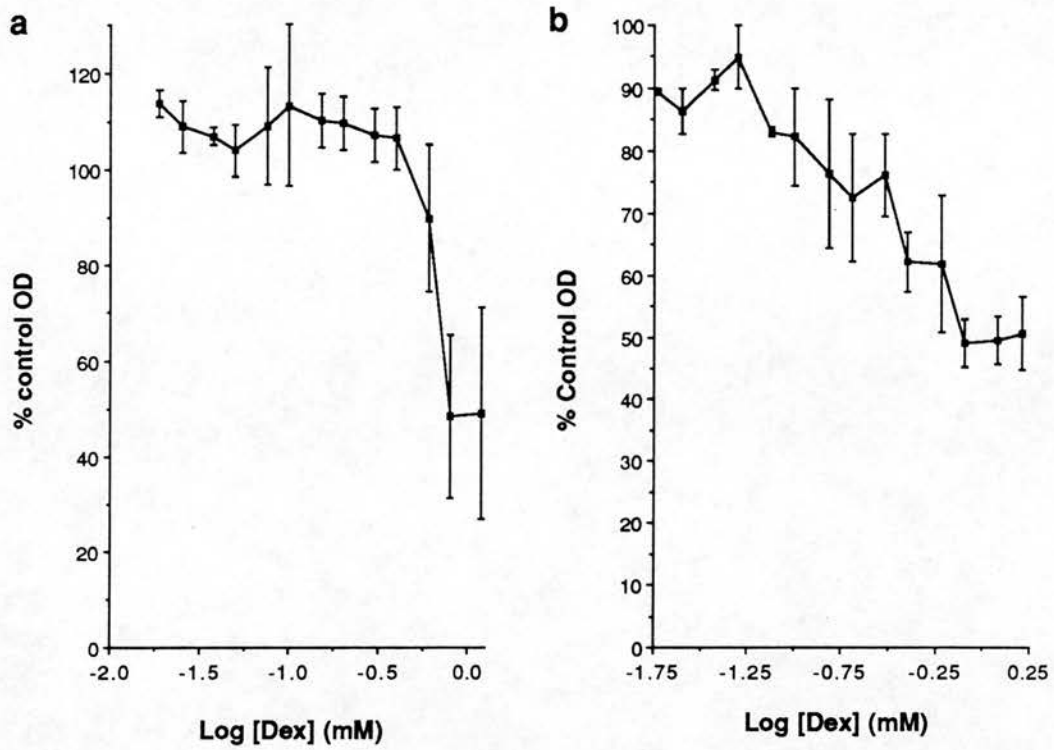
Toxicity of phenobarbital towards HepG2 and NCI H322 cells.



Plates were set up as described previously, plating out 10,000 HepG2 cells or 2500 NCI H322 cells per well in a volume of 180µl. The cells were allowed to adhere overnight and then 20µl of a 10x concentrated stock of PB in serum-free medium was added to give final concentrations of PB in the range 0 - 20mM. The plates were incubated for 5 more days then MTT was added and the plates developed as described.
(a) Toxicity towards HepG2 cells.
(b) Toxicity towards NCI H322 cells.

Figure 4.11.

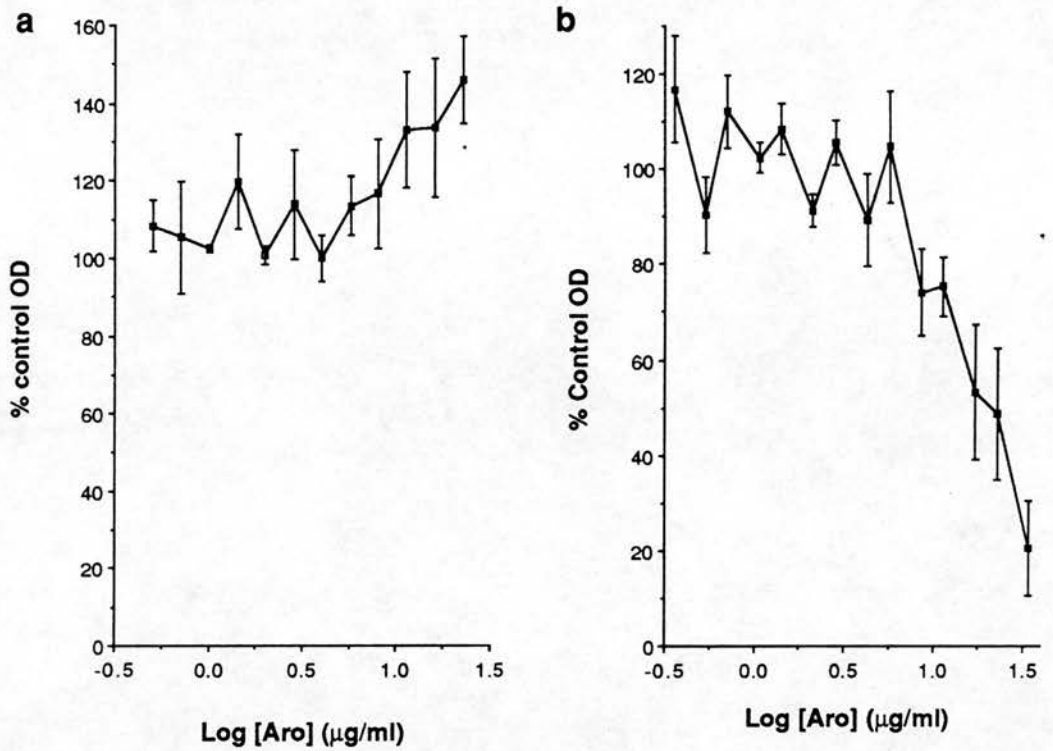
Toxicity of dexamethasone towards HepG2 and NCI H322 cells.



Plates were set up as described previously, plating out 10,000 HepG2 cells or 2500 NCI H322 cells per well in a volume of 180µl. The cells were allowed to adhere overnight and then refed with solutions of Dex in complete medium giving final concentrations of Dex in the range 0 - 1.6mM. The plates were incubated for 5 more days then MTT was added and the plates developed as described.
(a) Toxicity towards HepG2 cells.
(b) Toxicity towards NCI H322 cells.

Figure 4.12.

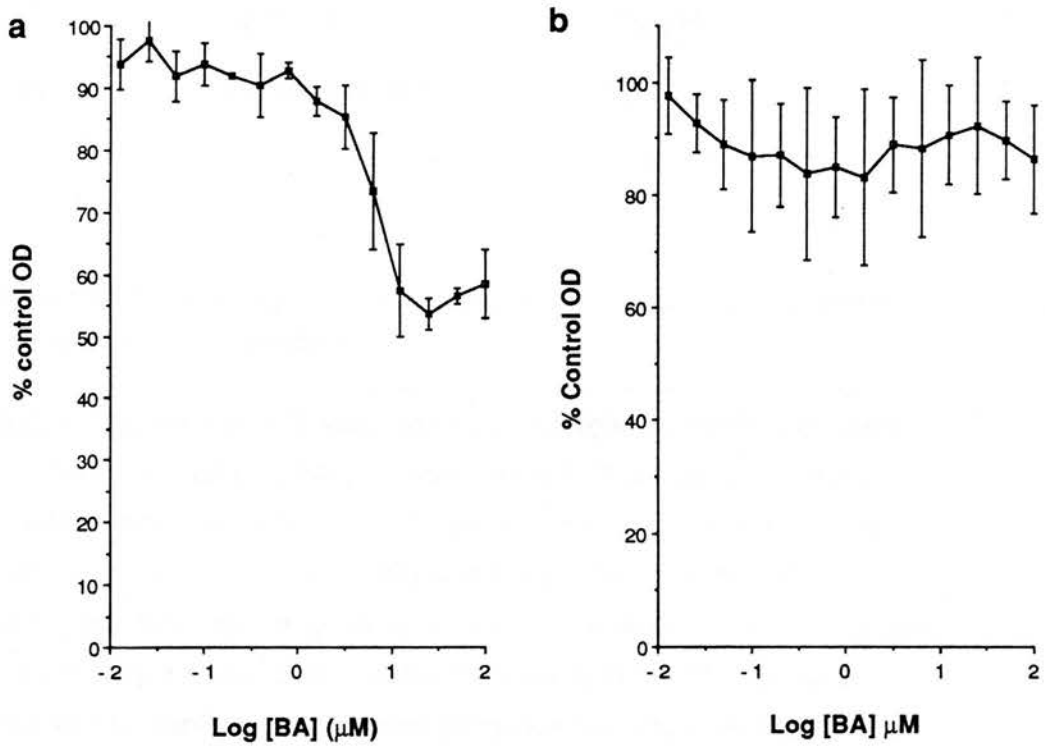
Toxicity of Aroclor 1254 towards HepG2 and NCI H322 cells.



Plates were set up as described previously, plating out 10,000 HepG2 cells or 2500 NCI H322 cells per well in a volume of 180µl. The cells were allowed to adhere overnight and then refed with solutions of Aroclor 1254 in complete medium giving final concentrations of Aroclor 1254 in the range 0 - 22.8µg/ml. The plates were incubated for 5 more days then MTT was added and the plates developed as described.
(a) Toxicity towards HepG2 cells.
(b) Toxicity towards NCI H322 cells.

Figure 4.13.

Toxicity of benzanthracene towards HepG2 and NCI H322 cells.



Plates were set up as described previously, plating out 10,000 HepG2 cells or 2500 NCI H322 cells per well in a volume of 180μl. The cells were allowed to adhere overnight and then refed with solutions of BA in complete medium giving final concentrations of BA in the range 0 - 100μM. The plates were incubated for 5 more days then MTT was added and the plates developed as described.
(a) Toxicity towards HepG2 cells.
(b) Toxicity towards NCI H322 cells.

Table 4.1.

Toxicity of P-450-inducing agents towards HepG2 and NCI H322 cells.

	IC_{50}	
	HepG2	**NCI H322**
Inducing agent:		
PB	4.2 mM	4.0 mM
Dex	800 μM	800 μM
Aroclor 1254	increased growth	22.4 μg/ml
BA	> saturation (30μM)	>saturation (30μM)

IC_{50} is defined as the concentration of inducing agent which causes a 50% reduction in the OD obtained after MTT treatment.

(a) Phenobarbital: Surprisingly, PB was cytotoxic at the higher concentrations used in induction experiments, causing 50% reduction in final OD at a concentration of about 4mM with both cell lines (Figure 4.10). However, there was no reduction in OD at the concentration used in the majority of induction experiments (1mM).
(b) Dexamethasone: Dex was not cytotoxic to HepG2 cells except at almost saturated concentrations (Figure 4.11(a)). Slight cytotoxicity towards NCI H322 cells was observed, but as with HepG2 cells 50% cytotoxicity was not reached until the concentration of drug was near to saturation (Figure 4.11(b)). Dex was not toxic to either cell line at the concentration used in induction experiments (10μM).
(c) Aroclor 1254: Aroclor 1254 consists of a mixture of various polychlorinated biphenyl compounds, so the concentrations used are quoted in micrograms per millilitre; they were in the same range as the concentrations of BA tested. Surprisingly, Aroclor 1254 increased the rate of growth of HepG2 cells, the increase being significant ($p<0.05$) at the higher concentrations tested (Figure 4.12(a)). This agent was slightly cytotoxic towards NCI H322 cells, causing 50% toxicity at around 20μg/ml, but no cytotoxicity was observed over the range of concentrations (3 - 9 μg/ml) used in induction experiments (Figure 4.12(b)).
(d) 1,2-Benzanthracene: BA was slightly toxic towards HepG2 cells at the higher

concentrations tested; the cytotoxicity curve levelled out at about 60% cell survival in the presence of saturated BA (Figure 4.13(a)). BA was not cytotoxic towards NCI H322 cells at any concentration tested (Figure 4.13(b)).

4.4. Effects of benzanthracene pretreatment on susceptibility of cells to benzo(a)pyrene and cyclophosphamide cytotoxicity.

4.4.1. Effects on benzo(a)pyrene toxicity.

Cells were treated with BA (13μM) or solvent (DMSO) alone for 24 hours prior to plating out on MTT plates. The treatment was continued for a further 24 hours as the cells were allowed to settle on the plates, then the inducing agent was removed and complete medium containing B(a)P was added. The remainder of the MTT assay was carried out as described previously. In order to ensure that induction had been successful, excess cells were retained and their EROD activity measured. The relative plating efficiency of the cells was assessed by comparing the "100%" OD reading (ie. that of cells treated with inducing agent or solvent but not B(a)P) with a control in which cells were plated out in the absence of any additions. This is expressed as "plating efficiency" in Figures 4.14, 4.15, 4.17 and 4.18.

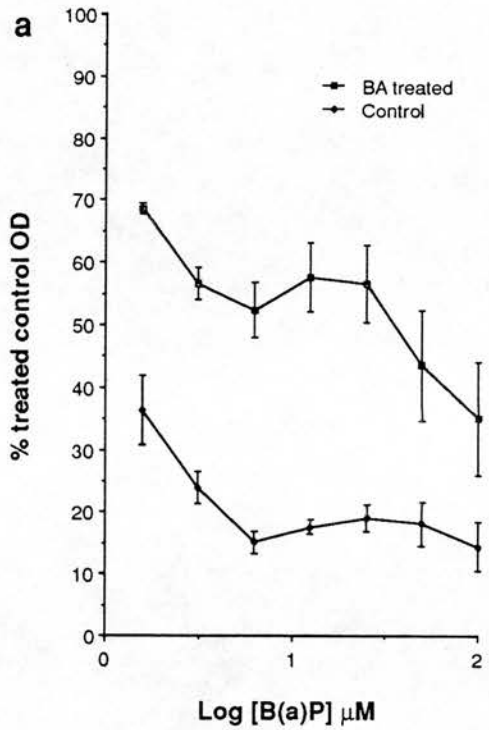
(a) Effect of pretreatment with BA on the susceptibility of HepG2 cells to benzo(a)pyrene cytotoxicity: Unexpectedly, pretreatment of HepG2 cells with BA appeared to reduce the susceptibility of the cells to the cytotoxic effect of B(a)P (Figure 4.14). It should be noted, however, that because BA treatment reduced the plating efficiency of the cells, the actual ODs (and hence cell numbers) after B(a)P exposure were very similar; the apparent protection due to BA treatment was a result of the lower absorbance of the BA-treated control compared with the DMSO-treated control. Figure 4.14 shows that BA-treatment gave approximately 2-fold protection against B(a)P cytotoxicity if the cell survived BA treatment.

(b) Effect of pretreatment with BA on the susceptibility of NCI H322 cells to benzo(a)pyrene cytotoxicity: B(a)P was not cytotoxic to either pretreated or untreated NCI H322 cells (Figure 4.15). Induction did reduce the plating efficiency of NCI H322 cells, but to a much smaller extent than was the case with HepG2 cells.

(c) Effect of duration of exposure to B(a)P on the susceptibility of HepG2 cells to its cytotoxicity: A possible reason for the failure of pretreatment with BA to increase HepG2 cells' susceptibility to B(a)P cytotoxicity was that treatment with

Figure 4.14.

Effect of pretreatment of HepG2 cells with benzanthracene on their susceptibility to the cytotoxic effects of benzo(a)pyrene.



(b)

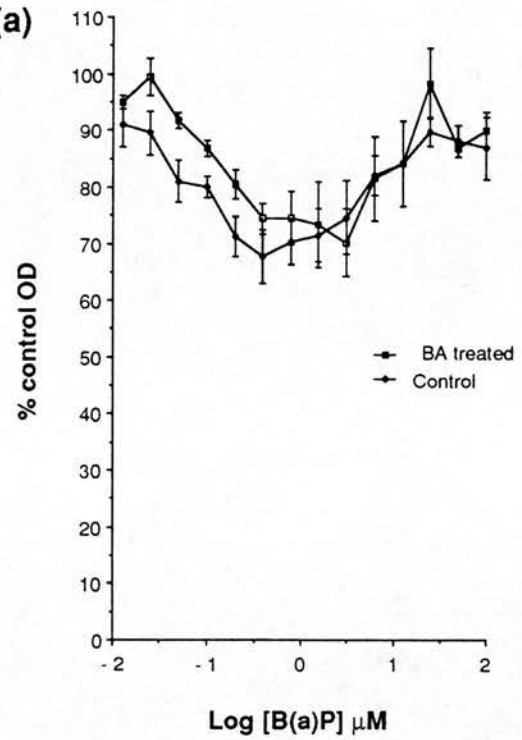
	Treatment		
	Control	**BA**	
EROD Activity (pmol/min/mg)	2.75 ± 0.25	61.7 ± 15.2	BA > C ($p = 0.057$)
Plating Efficiency (%)	95.3 ± 4.6	32.4 ± 2.8	BA < C ($p < 0.001$)

Confluent flasks of HepG2 cells were treated with BA (13μM) or DMSO alone (control) for 24 hours, then harvested and used to set up 96-well plates as described previously, plating out 10,000 cells per well. The remainder of the cells were retained for measurement of EROD activity. The cells were allowed to adhere overnight in the presence of BA or solvent and then refed with complete medium containing B(a)P over the concentration range 0 - 100μM. The plates were incubated for a further 4 days before developing as described previously. Percentage of control OD (ie. the OD of wells containing cells treated only with inducing agent) was calculated taking cells treated with BA or solvent as the control. Plating efficiency was calculated as the percentage OD in wells treated with BA or solvent compared with that in wells receiving no treatment.
(a) Toxicity of B(a)P towards BA- and DMSO-treated cells.
(b) Effect of BA-treatment on EROD activity and plating efficiency of the cells.

Figure 4.15.

Effect of pretreatment of NCI H322 cells with benzanthracene on their susceptibility to the cytotoxic effects of benzo(a)pyrene.

(a)



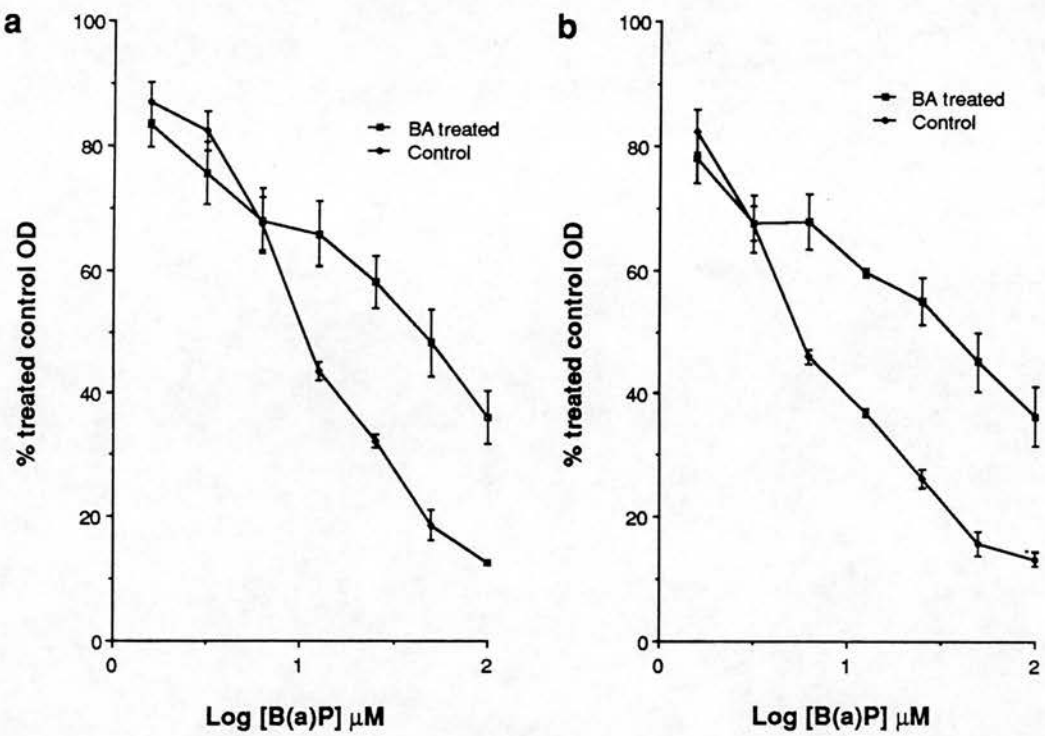
(b)

	Treatment		
	Control	**BA**	
EROD Activity (pmol/min/mg)	0.64 ± 0.11	5.61 ± 0.66	BA > C (p = 0.002)
Plating Efficiency (%)	106.0 ± 4.2	81.0 ± 3.0	BA < C (p < 0.008)

Confluent flasks of HepG2 cells were treated with BA (13μM) or DMSO alone (control) for 24 hours, then harvested and used to set up 96-well plates as described previously, plating out 10,000 cells per well. The remainder of the cells were retained for measurement of EROD activity. The cells were allowed to adhere overnight in the presence of BA or solvent and then refed with complete medium containing B(a)P over the concentration range 0 - 100μM. The plates were incubated for a further 4 days before developing as described previously. Percentage of control OD (ie. the OD of wells containing cells treated only with inducing agent) was calculated taking cells treated with BA or solvent as the control. Plating efficiency was calculated as the percentage OD in wells treated with BA or solvent compared with that in wells receiving no treatment.
(a) Toxicity of B(a)P towards BA- and DMSO-treated cells.
(b) Effect of BA-treatment on EROD activity and plating efficiency of the cells.

Figure 4.16.

Effect of duration of exposure to benzo(a)pyrene on its cytotoxic effect towards pretreated and control HepG2 cells.



Confluent flasks of HepG2 cells were treated with BA (13μM) or DMSO alone (control) for 24 hours, then harvested and used to set up 96-well plates as described previously, plating out 10,000 cells per well. The remainder of the cells were retained for measurement of EROD activity. The cells were allowed to adhere overnight in the presence of BA or solvent and then refed with complete medium containing B(a)P over the concentration range 0 - 100μM. The plates were incubated for either (a) 3 hours or (b) 6 hours then refed with complete medium without B(a)P and incubated for a further 4 days before developing as described previously. Percentage of control OD (ie. the OD of wells containing cells treated only with inducing agent) was calculated taking cells treated with BA or solvent as the control. N.B. The results of these experiments may be directly compared with those of the experiments shown in Figure 4.14, which were carried out at the same time. The EROD activities of the cells are also shown in Figure 4.14.

B(a)P itself induced MC_{1b} expression, so that after a sufficient length of exposure control and pretreated cells contained equally high levels of AHH activity. The effects of reducing the duration of exposure to B(a)P on the susceptibility of HepG2 cells to its cytotoxicity was examined (Figure 4.16). Cells were treated with BA or DMSO as described then exposed to B(a)P for 3, 6, or 96 hours (Figures 4.14 and 4.16). The experiments shown in Figure 4.14 and Figure 4.16 were performed at the same time; the results of these experiments are summarised in Table 4.2. The results of this comparison showed that the susceptibility of control cells to B(a)P toxicity was dependent on the duration of exposure, but the BA-treated cells were equally resistant to cytotoxicity over the three time periods tested.

Table 4.2.

Effect of duration of exposure to B(a)P on the susceptibility of HepG2 cells to its cytotoxic effects.

	IC_{50}	
	Control cells	**BA-treated cells**
Duration of exposure to B(a)P:		
3 hours	11.2μM	50μM
6 hours	5.6μM	40μM
96 hours	<1.56μM	40μM

IC_{50} is defined as the concentration of B(a)P which causes a 50% reduction in the OD obtained after MTT treatment.

(d) EROD activity at the time of exposure to B(a)P: In previous experiments, the EROD activity of the whole population of cells was measured at the time of plating. In view of the low plating efficiency of the BA-treated HepG2 cells, a possible reason for apparent protection by BA-treatment against B(a)P cytotoxicity is that the cells which express MC_{1b} failed to adhere to the plate, so that the population exposed to B(a)P really had low AHH activity. Experiments were carried out to test this hypothesis. Two flasks were treated with BA and two taken as controls and after 24 hours all the flasks were harvested, one from each pair being retained for 7-EROD

measurement and the other placed in a fresh flask. After a further 24 hours, ie. at the time at which the drug would be added to an MTT assay, these two flasks were harvested and prepared for EROD measurement; the results are shown in Table 4.3. The results showed that cells treated with BA maintained their EROD activity at the time of exposure to B(a)P; indeed, EROD activity was further increased compared with that at the time of plating.

Table 4.3.

Comparison of EROD activity of HepG2 cells at time of setting up MTT assay and at time of exposure to B(a)P.

	EROD activity (pmol/min/mg)	
	Control cells	**BA-treated cells**
Time after BA treatment:		
24 hours (time of plating out)	2.77 ± 0.74	63.1 ± 14.8
48 hours (time of exposure to B(a)P)	2.27 ± 0.55	116.4 ± 26.5

4.4.2. Effects on cyclophosphamide toxicity.

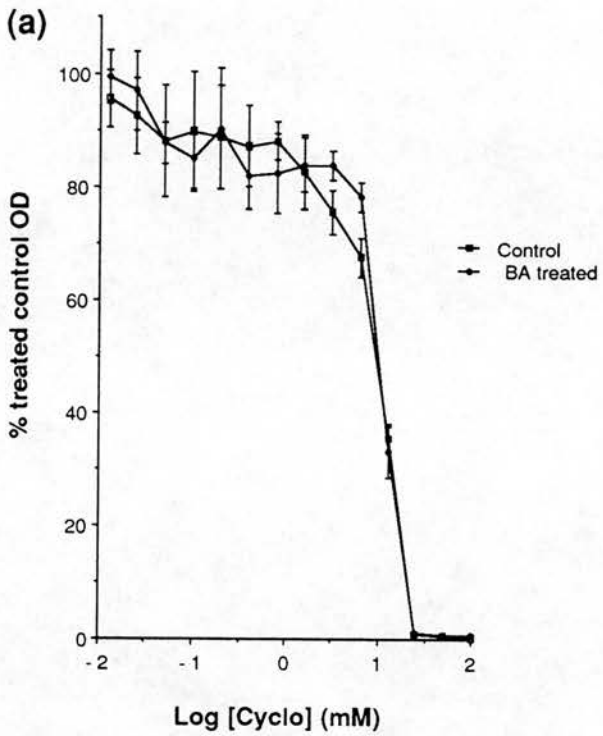
Experiments to test the effects of BA pretreatment on the susceptibility of HepG2 and NCI H322 cells to cyclophosphamide cytotoxicity were carried out in the same way as the experiments using B(a)P except that cyclophosphamide was sufficiently soluble in complete medium to be dissolved directly.

(a) Effect of BA treatment on HepG2 cells' susceptibility to cyclophosphamide:

Cyclophosphamide was toxic to both BA-treated and control HepG2 cells, having an IC_{50} of 8mM (Figure 4.17). As in the experiments using B(a)P, the plating efficiency of the BA-treated cells was significantly lower in the BA-treated cells than in the control cells, but when the results were calculated taking cells treated with BA or solvent only as the 100% value the cytotoxicity curves for BA-treated and control cells overlapped exactly. This showed that BA treatment had no effect on susceptibility to cyclophosphamide and indicates that the cells surviving BA

Figure 4.17.

Effect of pretreatment of HepG2 cells with benzanthracene on their susceptibility to the cytotoxic effects of cyclophosphamide.



(b)

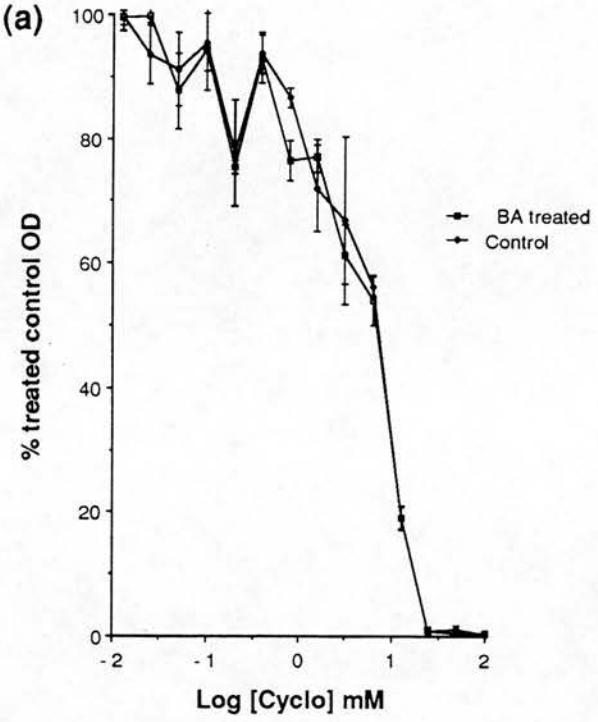
	Treatment		
	Control	**BA**	
EROD Activity (pmol/min/mg)	2.17 ± 0.66	26.7 ± 4.7	BA > C (p = 0.007)
Plating Efficiency (%)	89.4 ± 5.7	48.3 ± 5.2	BA < C (p < 0.006)

Confluent flasks of HepG2 cells were treated with BA (13μM) or DMSO alone (control) for 24 hours, then harvested and used to set up 96-well plates as described previously, plating out 10,000 cells per well. The remainder of the cells were retained for measurement of EROD activity. The cells were allowed to adhere overnight in the presence of BA or solvent and then refed with complete medium containing cyclophosphamide over the concentration range 0 - 100mM. The plates were incubated for a further 5 days before developing as described previously. Percentage of control OD (ie. the OD of wells containing cells treated only with inducing agent) was calculated taking cells treated with BA or solvent as the control. Plating efficiency was calculated as the percentage OD in wells treated with BA or solvent compared with that in wells receiving no treatment.
(a) Toxicity of cyclophosphamide towards BA- and DMSO-treated cells.
(b) Effect of BA-treatment on EROD activity and plating efficiency of the cells.

Figure 4.18.

Effect of pretreatment of NCI H322 cells with benzanthracene on their susceptibility to the cytotoxic effects of cyclophosphamide.

(a)



(b)

	Treatment		
	Control	**BA**	
EROD Activity (pmol/min/mg)	0.14 ± 0.04	0.77 ± 0.14	BA > C (p = 0.013)
Plating Efficiency (%)	98.8 ± 2.5	88.5 ± 8.8	N / S

Confluent flasks of NCI H322 cells were treated with BA (13μM) or DMSO alone (control) for 24 hours, then harvested and used to set up 96-well plates as described previously, plating out 10,000 cells per well. The remainder of the cells were retained for measurement of EROD activity. The cells were allowed to adhere overnight in the presence of BA or solvent and then refed with complete medium containing cyclophosphamide over the concentration range 0 - 100mM. The plates were incubated for a further 5 days before developing as described previously. Percentage of control OD was calculated taking cells treated with BA or solvent as the control. Plating efficiency was calculated as the percentage OD in wells treated with BA or solvent compared with that in wells receiving no treatment.
(a) Toxicity of cyclophosphamide towards BA- and DMSO-treated cells.
(b) Effect of BA-treatment on EROD activity and plating efficiency of the cells. It should be noted that the observed activities were very low in this experiment.

treatment did not have improved resistance to all cytotoxic agents.

(b) Effect of BA treatment on NCI H322 cells' susceptibility to cyclophosphamide: Cyclophosphamide was toxic to both BA-treated and control NCI H322 cells, having an IC_{50} of 8mM, the same as the IC_{50} towards HepG2 cells (Figure 4.18). As with HepG2 cells, BA treatment had no effect on the susceptibility of NCI H322 cells to cyclophosphamide cytotoxicity. However, firm conclusions cannot be drawn from this experiment because the EROD activities of both control and BA-treated cells were very low, possibly due to prolonged storage of the samples. The activity of the BA-treated samples, though low, was significantly higher than that of the control.

4.5. Discussion.

In this section of the project, the MTT assay was characterised and used to assess the toxicity of P-450-inducing agents towards cultured cells. The MTT assay is useful in studies of this kind because it is reproducible and simple to perform. Many compounds may easily be assayed in a relatively short time, and using a simple modification of the published method hydrophobic compounds may be tested up to saturated concentrations in the culture medium. The most serious disadvantage of the MTT assay is that it cannot distinguish between cytotoxic and cytostatic effects, since the absorbance reading is related only to the absolute number of cells present at that time, and not to their rate of growth. The experiments described in Section 4.3 showed that the cell lines HepG2 and NCI H322 were appropriate for use in the MTT assay since they grew consistently on 96-well plates; the relationship between the OD obtained after treatment with MTT and the cell number present approximated to linearity. By plating out appropriate cell numbers the cells were kept in logarithmic growth to the end of the assay but sufficiently high OD readings were obtained for reproducible measurement of cytotoxicity. As expected, the majority of the P-450 inducing agents tested caused little toxicity to either of the cell lines, although PB caused 50% cell death at about 4mM. This was surprising, particularly in view of the reported beneficial effect of 3mM PB on hepatocyte survival in primary culture (Miyazaki et al (1985)). Aroclor 1254 actually enhanced the rate of growth of HepG2 cells relative to control cells treated with DMSO alone: the mechanism of this effect is unknown and merits further study.

Part of the aim of the present study was to establish whether induction of P-450s in cultured cells could increase their sensitivity to the cytotoxicity of compounds which

undergo P-450 mediated metabolic activation. A precedent does exist, in that pretreatment of HepG2 cells with 3-MC was reported to increase their sensitivity to aflatoxin B_1 toxicity (Herweijer et al (1988)). However, in the experiments described above this was not the case; indeed, pretreatment of HepG2 cells with BA appeared to reduce their susceptibility to B(a)P cytotoxicity. There are a number of possible explanations for this effect. The toxicity of B(a)P could be a direct effect, perhaps occurring as a result of intercalation into DNA, or an indirect effect arising from metabolic activation of the molecule to form toxic metabolites. Protection from B(a)P toxicity could arise as a result of increased detoxification or increased activation, either of which may be mediated by P-450s. Efficient DNA repair or free-radical scavenging systems could also be involved.

One possibility is that BA treatment induced a P-450 which metabolised B(a)P rapidly to form a non-toxic product leading to efficient detoxification within the cell. Such a mechanism has been proposed for the protective effect of 3-MC treatment against liver necrosis caused by bromobenzene (Jollow and Smith (1977)). Bromobenzene is metabolised via two P-450-dependent pathways, a PB-inducible route leading to increased cytotoxicity and a 3-MC inducible route thought to produce bromobenzene-2,3-epoxide which is efficiently detoxified by EH (Zampaglione et al (1973)). Induction with PB potentiates the development of liver necrosis due to bromobenzene, whilst induction with 3-MC, by altering the route of bromobenzne metabolism, protects the liver from cytotoxicity. Such a mechanism would not be consistent with the known role of P-450s in the MC-inducible family in the metabolism of B(a)P, but the roles of human isozyme MC_{1b} are not well characterised and could differ from those of the equivalent isozyme in other species.

A second possibility, suggested by the low plating efficiency of HepG2 cells in the presence of BA and the fact that approximately 60% of HepG2 cells were resistant to BA cytotoxicity, is that cells which express high levels of MC_{1b} were killed by the initial BA treatment so that the population encountering B(a)P actually expressed only low AHH activity. Such a phenomenon would be analogous to the selection system used by Hankinson to isolate B(a)P resistant variants of the mouse hepatoma cell line Hepa-1. In Hankinson's experiments B(a)P itself was used as the selective agent whereas in the present case BA treatment appeared to result in selection of a cell population resistant to subsequent exposure to B(a)P. Hankinson found that his

B(a)P-resistant clones of Hepa-1 cells were resistant to induction of AHH activity by BA because of defects in either the MC_{1b} structural gene or the Ah receptor (Legraverend et al (1982), Miller et al (1983), Israel et al (1983)). If the selection of B(a)P-resistant HepG2 cells by BA was truly analogous to Hankinson's system the cells which survived BA-treatment would be expected to contain low levels of AHH actvity at the time of exposure to B(a)P. This hypothesis was tested in the experiment shown in Table 4.3, which showed that no such effect occured; the cells which survived plating out continued to be induced by BA, expressing high levels of EROD (and presumably AHH) activity at the time at which they would have been exposed to B(a)P. These results contrast with those obtained by Hankinson, and other explanations for the resistance of these cells to B(a)P must be considered. In order to investigate further, the experiment should be repeated exposing the cells to a lower, non-toxic, concentration of BA in order to establish whether resistance to B(a)P is still observed. This would indicate whether the observed resistance was the result of selection of a population of cells which was already resistant to B(a)P or induction of a protective factor such as a detoxifying enzyme within the cells.

The third, and most likely, possibility is that BA either induced detoxifying enzymes in the HepG2 cells or selected a population of cells which already contained high levels of detoxifying activities. The primary metabolites of B(a)P undergo further metabolism by a variety of enzymes including EH, glutathione-S- transferases (GSTs), UDP-glucuronyl transferases (UDPGTs) and sulphotransferases. Many detoxifying enzymes are inducible in vivo by PAHs; these include EH, which is involved in both the activation and detoxification of B(a)P (Gelboin (1980)) and conjugating enzymes. The GSTs comprise a multigene family of dimeric enzymes which conjugate glutathione to reactive electrophiles producing readily excreted hydrophilic conjugates (Mannervik (1985)). Several GSTs were shown to be 3-MC-inducible at both the enzymic and mRNA levels (Hales and Neims (1977), Pickett et al (1984)); this induction appeared to occur at the level of increased transcription of the Ya and Yb gene families (Ding and Pickett (1985)). Recent studies confirmed that the Ya and Yb subunits were induced after treatment of rats with BA, but the Yf subunit was not BA-inducible (Dolan et al (1988)). The UDPGTs also constitute a multigene family of enzymes which, like the GSTs, are involved in conjugation reactions leading to the production of hydrophilic and readily excreted metabolites, in this case glucuronides (Burchell (1981)). It was

shown that 3-MC treatment of rats led to elevation of UDPGT activity towards phenolic substrates, including some primary metabolites of B(a)P (Bock et al (1973), Wishart (1978)). It was proposed that the major 3-MC-inducible UDPGT is important in protecting the cell from oxygen free-radical generation due to redox cycling of B(a)P quinone and quinol metabolites (Bock et al (1980), Lilienblum et al (1985)). However, reduced product inhibition of P-450-mediated B(a)P metabolism as a result of increased UDPGT activity can actually increase covalent binding of B(a)P metabolites to DNA (Fahl et al (1978)). A model linking induction of P-450s and detoxifying enzymes by Ah ligands was proposed by Prochaska et al (1985, 1988), who suggested that some inducing agents induce both P-450s and detoxifying enzymes via the Ah receptor and are then metabolised to electrophilic products which continue to induce detoxifying enzymes but not P-450s. In such a situation, the ability to induce detoxifying enzymes would be maintained for longer than the ability to induce P-450s leading to a predominance of detoxifying enzymes and hence to efficient clearance of toxic metabolites. In this case, pretreatment of the cell with a "bifunctional" inducer (ie. one which can induce both P-450s and detoxifying enzymes) might protect the cell from further chemical insult.

It is not clear whether protection against B(a)P toxicity by induction of detoxifying enzymes is possible in HepG2 cells. It is thought that HepG2 cells contain efficient detoxifying systems; they metabolised DMBA as rapidly as hamster embryo cells but the resulting mutagenicity towards V79 cells was much lower in HepG2-mediated than in hamster embryo cell-mediated assays (Diamond et al (1984), DiGiovanni et al (1984)). However, the role of UDPGTs in HepG2 cells is equivocal: the cells were shown to be capable of glucuronidating 7-EC (Dawson et al (1985)) but other reports stated that the cells contained little glucurinidation or sulphation activity (Diamond et al (1980)), or that they contained the same level as freshly isolated human.hepatocytes, but were refractory to UDPGT induction by BA (Grant et al (1988)). The reason for this apparent discrepancy may be that in the first report the conjugation of B(a)P metabolites was studied whilst in the second the substrates used were phenolphthalein and 1-naphthol. Little is known about the regulation of GSTs in HepG2 cells: the cell line has been shown by Western blot analysis and enzyme activity to express GSTs from all three gene families (A.D. Lewis, PhD thesis (1988)), but GST inducibility in this cell line is poorly understood.
Further experiments would show whether induction of GSTs could protect a certain cell population against B(a)P toxicity as a result of pretreatment with BA.

In these experiments the cell line NCI H322 was resistant to the cytotoxicity of B(a)P, regardless of whether cells had been pretreated with BA. This result contrasted with a recent report that NCI H322 cells suffer 50% cell death following treatment with 10μM B(a)P (Keifer et al (1988)). The difference may be a function of the different methods used (the MTT assay versus measurement of cellular protein and cloning efficiency); this point requires further clarification. The major route of detoxification of B(a)P metabolites in human lung tumours is unclear: in normal human peripheral lung tissue, sulphation appears to be an important conjugation route (Mehta and Cohen (1979)), but a number of human lung tumour-derived cell lines conjugate B(a)P metabolites to UDP-glucuronic acid rather than sulphate (Gibby et al (1981)). The pathway of B(a)P detoxification in NCI H322 cells does not appear to involve glucuronidation; there is some debate concerning the roles of sulphotransferase- and GST- mediated detoxification of B(a)P metabolites in this cell line (Wiebel et al (1986), Keifer et al (1988)). Repair of DNA lesions caused by B(a)P metabolites could also be important in NCI H322 cells' resistance to toxicity: the human lung tumour-derived cell line A549 was shown to be able to eliminate some, but not all, B(a)P diol epoxide-induced DNA lesions within 72 hours (Cerutti et al (1978), Feldman et al (1978)).

Cyclophosphamide was toxic to both HepG2 and NCI H322 cells, demonstrating that they had the ability to activate this drug, in agreement with the work of Dearfield et al (1983, 1986). Treatment with BA had no effect on susceptibility to cyclophosphamide, suggesting either that MC_{1b} is not involved in cyclophosphamide activation, or that both activating and detoxifying systems were equally induced by BA. It should be noted that the level of EROD activity in the NCI H322 cells used for this experiment were extremely low, making it difficult to interpret the results obtained. In the experiment using HepG2 cells, however, a high level of induced EROD activity was observed; this had no effect on the cells' sensitivity to cyclophosphamide. The fact that HepG2 cells surviving BA treatment did not have altered susceptibility to cyclophosphamide shows that protection against B(a)P toxicity due to BA treatment was not a universal phenomenon affecting susceptibility to all cytotoxic agents.

Part of the reason for carrying out these studies was the hope that induction of P-450-dependent metabolic activation of anticancer drugs would increase the

sensitivity of refractory human tumours to certain chemotherapeutic agents. The idea that induction of P450s in the liver might improve the therapeutic response to drugs such as cyclophosphamide arose in the late 1960s. However, this prediction soon proved to be erroneous, when it was shown that induction with PB, whilst increasing the rate of metabolism of cyclophosphamide, did not affect its therapeutic efficiency. Similarly, treatment of rats with SKF 525A to inhibit cyclophosphamide metabolism failed to alter its therapeutic efficiency (Sladek (1971,1972 a,b), Field et al (1973)). It was proposed that the reason for these effects was a concomitant change in the clearance of alkylating metabolites (Sladek (1973)). A change in the pathway of P-450-mediated metabolism to dechlorination rather than 4-hydroxylation after PB treatment has also been suggested (Struck et al (1984)).

In this work, a slightly different approach was taken in that the aim was to induce P-450-dependent metabolic activation in the tumour itself, in the hope that the short-lived reactive intermediates generated would attack the tumour cells themselves but not enter the bloodstream; the results, however, were not as predicted. Pretreatment with BA caused, if anything, a decrease rather than an increase in susceptibility to cytotoxicity. This finding illustrates the importance of achieving a complete understanding of the routes of metabolism of a particular compound in the cell type to be studied before attempting to predict the outcome of a change in P-450-dependent metabolism. The idea of inducing P-450-dependent drug activation as an approach to increasing the sensitivity of tumours to chemotherapy does, however, merit further consideration. Human tumour-derived cell lines which are responsive to induction of P-450s would be of value in the early stages of such a project, although it should be noted that the routes of metabolism of drugs may differ between the tumour and the cell line, and indeed between different tumours. A number of requirements would need to be fulfilled in order for this approach to chemotherapy to find success: a thorough understanding of the metabolic pathways followed by the drug in question is the most important, but the actions of the inducing agent should also be well characterised and it should only induce enzymes involved in the activation of the candidate drug. A means must be devised to deliver the inducing agent directly to the tumour, avoiding induction of drug activation in the liver, and the inducing agent must pass the legal criteria for use as a drug. These targets will be difficult to achieve, but are worth aiming for if the outcome is an improvement in the reponse of refractory tumours to chemotherapy.

Chapter 5.
Effects of inflammatory mediators on P-450 expression.

5.1. Aims.

Previous reports showed that intraperitoneal doses of endotoxin suppressed hepatic P-450-dependent monooxygenase reactions; these studies were limited in that the expression of specific isozymes was not studied. A preliminary study carried out in this laboratory before the present project indicated that endotoxin can profoundly and differentially affect mouse hepatic cytochrome P-450 isozymes in uninduced animals and animals treated with PB or 3-MC (Robert Lindsay, Final Year Undergraduate Project (1985), D.J. Adams, personal communication).

The aims of this section of the present project were

(i) To extend observations indicating that endotoxin has differential effects on P-450 expression in the mouse liver.

(ii) To use the human lung tumour-derived cell line NCI H322 to study the effects on pulmonary P-450 expression of cytokines implicated in response to endotoxin.

5.2. Introduction to cytokines.

A variety of mediators have been implicated in the effects of endotoxin. The aim of this section of the project was to try to establish which of these may be responsible for endotoxin's effects on P-450-dependent activities. The effects of endotoxin are mediated by a number of protein factors which were initially known as lymphokines (if derived from lymphocytes) or monokines (if derived from monocytes). They are now classified as cytokines since some are synthesised by more than one cell type (Harrison and Campbell (1988)). Many cytokines which interact with each other in complex patterns have now been identified; five were chosen for this project. These were tumour necrosis factor (TNF) and interleukin-1 (IL-1), which are thought to be the major mediators of the effects of endotoxin, and representatives of the three families of interferon, interferons alpha (ifn α), beta (ifn β) and gamma (ifn γ). The cell lineages from which they are derived are shown in Table 5.1.

Table 5.1.

Cellular sources of cytokines.

Cytokine	Abbreviation	Cell type of origin
Interleukin 1	IL-1	Monocyte/macrophage lineage Many other nucleated cell types
Tumour Necrosis Factor	TNFα	Monocyte/macrophage lineage
Interferon α	Ifn α	Leukocytes Fibroblasts produce some ifn α
Interferon β	Ifn β	Fibroblasts
Interferon γ	Ifn γ	T-lymphocytes

Summarised from Harrison and Campbell (1988).

5.2.1. Mediators of the effects of endotoxin: IL-1 and TNF.

The cytokines TNF and IL-1 are members of distinct families but have overlapping functions in mediating the effects of endotoxin. Both are the subject of intensive research because of their involvement in many aspects of inflammation and immunology (Beutler and Cerami (1988), Cerami and Beutler (1988), Dinarello (1986, 1988)). The properties of these cytokines and the evidence that they mediate the effects of endotoxin are discussed briefly below.

TNF, also known as cachectin, was discovered independently in two research areas. Studies on endotoxin-induced tumour necrosis identified a mediator called tumour necrosis factor (Shalaby et al (1986)), whilst studies concerning cachexia (wasting) suffered by patients with tumours and chronic infective diseases resulted in the discovery of cachectin (Beutler and Cerami (1986)). Protein purification and molecular cloning showed that these effects were mediated by the same protein, a polypeptide of molecular weight (Mr) 17,000. This highly conserved protein is synthesised as a prohormone, undergoing post- translational cleavage to release the mature protein which exists in dimeric, trimeric or pentameric forms. Its actions are mediated by high affinity receptors on target cells: TNF-sensitive cells have about 10,000 such receptors (dissociation constant (Kd) = 3×10^{-9}M).

Biosynthesis of TNF occurs in response to invasive stimuli, including parasites, viruses, bacteria and tumours; injection of endotoxin also induces TNF release. Evidence implicating TNF in the mechanism of action of endotoxin was derived from various observations. Injection of endotoxin into either experimental animals or humans led to a rapid increase in the serum concentration of TNF; endotoxin and TNF caused similar effects in healthy human volunteers (Waage (1987), Michie et al (1988a)). Injection of serum from endotoxin-treated C3H/HeN mice into endotoxin resistant C3H/HeJ mice led to the development of symptoms characteristic of endotoxin effects in sensitive mice, including diarrhoea, withdrawn appearance, anorexia, anaemia, fever and neutrophilia. These effects were also observed after injection of recombinant TNF (Bauss et al (1987)). Passive immunisation with a polyclonal antibody raised against TNF protected mice against the lethal effects of endotoxin (Beutler et al (1985), Flick and Gifford (1986)). Finally, when nude mice were injected with CHO cells bearing the TNF gene linked to the cytomegalo-virus promoter, they rapidly became cachectic whereas mice bearing CHO cell tumours carrying only the promoter remained healthy (Oliff et al (1987)).

Although there is considerable evidence that TNF can mediate some of the effects of endotoxin, controversy still surrounds its exact role in infection. Attempts to show altered serum TNF levels in humans following infection were inconclusive, and in experimental studies only low TNF levels were detected in mice during inflammation (Moldawer et al (1987)). The release of TNF into serum after endotoxin treatment appeared to be of very brief duration; the cytokine was rapidly cleared from the circulation and did not reappear after repeated endotoxin treatment (Waage (1987), Michie et al (1988b)). Studies on the regulation of TNF expression suggested that its synthesis was tightly and rapidly controlled both at the level of transcription and by RNA stabilisation (Beutler et al (1986)). These observations led to the proposal that if TNF is important in infection, this effect is confined to the earliest stage of the process and other factors take over within a few hours.

IL-1 has also been implicated in responses to infection and inflammation. This molecule too has had a confusing history, having been called endogenous pyrogen, lymphocyte activating factor and leukocyte endogenous mediator. Many stimuli induce IL-1 release, including antibody-antigen complexes, activated complement components, T-cell-derived factors including ifn γ, cell injury, phorbol esters and

calcium ionophores, as well as endotoxin (Martin and Resch (1988)). Many cell types are able to synthesise IL-1 in response to these stimuli, the major source being the macrophage. For this project it is of interest that cultured human lung fragments can also release IL-1 (Bochner et al (1987a)).

Two IL-1 forms with different isoelectric points exist: IL-1α has an isoelectric point of 5.0 whilst that of IL-1β is 7.0. The major form of IL-1 released in response to endotoxin appears to be IL-1β, which is synthesised as a prohormone (Mr = 31,000) then cleaved to form the mature IL-1 found in serum (Mr = 17,500). A proportion of IL-1 remains membrane bound and this component may be important in the cytokine's local effects. IL-1 lacks a classical signal cleavage site and is instead cleaved by serine proteases; it has been suggested that such cleavage may be to some degree "accidental" and the major role of IL-1 may a local one involving the membrane bound form. However, there is little direct evidence to support this hypothesis and circulating IL-1 has a variety of effects, mediated by binding to receptors on sensitive cells. Two classes of IL-1 receptors exist: a high affinity, low abundance class (100 - 4000 sites per cell, Kd = 5×10^{-12}M) and a lower affinity, high abundance class (15,000 sites per cell, Kd = 4×10^{-10}M).

IL-1 has multiple roles in the inflammatory process, including induction of fever, possibly mediated via elevated production of prostaglandin E_2, wasting (similar to the effect of TNF), and induction of the acute phase response in the liver. IL-1 causes release of a number of other hormones, including endorphins, corticotrophin releasing factor, adrenocorticotrophic hormone and somatostatin. It also affects the serum levels of various metal ions, causing a decrease in levels of iron and zinc but elevating the level of copper. Induction of adrenocorticotrophic hormone by IL-1 leads to elevated steroid synthesis in the adrenal gland. This is an important feature of the response to endotoxin, in that the release of glucocorticoids as a result of endotoxin treatment forms a potential regulatory feedback loop preventing overexpression of molecules such as IL-1 during inflammation (Del Rey et al (1987)). The loop is thought to function as follows: endotoxin induces the release of IL-1 which causes the release of adrenocorticotrophic hormone and hence release of glucocorticoids from the adrenal gland (Besedovsky et al (1986)). These suppress the further production of IL-1, acting at two levels, inhibition of transcription and destabilisation of IL-1 mRNA (Bochner et al (1987b), Lee et al (1988)).

Glucocorticoids also regulate the expression of other cytokines, including TNF and interferon (Beutler et al (1986), Gessani et al (1988)). In view of the effects of glucocorticoids in P-450 regulation and the finding that IL-1 affects the hepatic response to glucocorticoid hormones (Hill et al (1986)), the synthetic glucocorticoid Dex was included in the group of mediators whose effect on P-450 induction in NCI H322 cells was studied.

5.2.2. Interferons.

Interferons (ifns) are another group of cytokines produced during viral infection, the acute phase response, and endotoxaemia. When first discovered, interferons were of great interest because of their anti-viral and possible anti-tumour effects; they were subsequently shown to mediate many aspects of cell growth and function as well as inflammation. Interferons were defined as "proteins which exert virus-non-specific antiviral activity, at least in homologous cells, through cellular metabolic processes involving synthesis of both RNA and protein" (Stewart et al (1980)). Three families of interferon exist: α (leukocyte), β (fibroblast) and γ (immune) interferon (Revel and Chebath (1986), Pestka et al (1987)). Members of all three classes have been purified and characterised, and representatives of each family cloned at the cDNA and genomic level (Knight (1984)).

The ifns α constitute a family of closely related peptides which have been purified from a number of normal and malignant leukocyte populations. They contain 165 or 166 amino acids, and have very similar amino acid compositions, rich in leucine, glutamine and glutamic acid, although there is considerable charge heterogeneity between members of this family. Four conserved cysteines, at amino acid positions 1, 29, 98/99 and 138/139 are thought to maintain the secondary structure of the protein by intramolecular disulphide bonding. The ifns α are glycosylated, probably via O-linkages, and therefore have heterogeneous molecular weights in the range 16,000 - 27,000. They appear to be monomeric in solution. The first interferon to be purified, from diploid fibroblasts, was ifn β. This was at first thought to be a single entity: a second ifn β was recently identified but its relationship to other interferons is still a subject of debate (Revel and Zilberstein (1987)). The "true" ifn β is a protein (Mr = 20,000) which shares considerable sequence homology with the ifns α. Conserved cysteines at positions 17, 29 and 141 of ifn β are

probably involved in intramolecular disulphide bonds; N-glycosylation of the molecule occurs at asparagine 80. Ifn β appears to be dimeric in solution. The third class of interferon, ifn γ, is classified as both an interferon and a lymphokine. It was first purified from peripheral blood lymphocytes, and comprises several forms (Mr =15,500 - 17,000, 20,000 and 25,000); these contain the same core peptide but are differently glycosylated. The Mr = 20,000 form is glycosylated only at asparagine 25 whilst the Mr = 25,000 form is also glycosylated at asparagine 97. Ifn γ may exist in dimeric and tetrameric forms in solution. Two classes of interferon receptor exist; the type I receptor binds both ifn α and ifn β, whilst the type II receptor is specific to ifn γ. The number of receptors per cell varies between about 1000 - 10,000, and both receptor types have dissociation constants of 10^{-9} - 10^{-11}M. The mechanism of the interaction between interferons and their receptors is not fully understood; it thought to be highly complex. Ifns α and β, for example, can exert different effects following interaction with the Type I receptor. The intracellular messengers involved in interferon action have not been identified, although it is thought that cyclic AMP may be involved in some of them.

Interferons are synthesised in response to a large number of different stimuli. Inducers of ifns α and β include viruses, double-stranded RNA, intracellular microorganisms, protozoans, pyran copolymers and polyvinyl sulphate as well as small molecules including kanamycin, tilorone, cycloheximide, turpentine and toluidine blue. Molecules which activate T-cells, such as antigens and mitogens, are inducers of ifn γ (Wietzerbin et al (1977), Preble and Friedman (1983), Ho (1984)). Endotoxin causes heterogeneous induction of both type I (α and β) and type II (γ) interferons. Induction of ifn γ in response to endotoxin is thought to be mediated by IL-1 and IL-2 (Maehara and Ho (1977), Le et al (1986)).

Interferons have many cellular effects, the best characterised being protection against viruses by induction of an "antiviral state". This is thought to involve an interferon-inducible protein kinase, the P1/eIF-2α protein kinase, which is located in ribosomes and may suppress the translation of viral RNAs. The so-called Mx protein may also be involved in the induction of the antiviral state. Interferons are involved in the regulation of cell growth and differentiation, possibly acting via a specialised enzyme system which synthesises 2'-5'-oligoadenylates; the role of

this class of oligonucleotides in cellular regulation is not fully understood (Taylor-Papadimitriou (1984), Moritz and Kirchner (1986)). Interferons have a number of effects on the immune system, including activation of macrophages and cytotoxic T-lymphocytes, stimulation of natural killer cell activity and regulation of the expression of the major histocompatibility antigens. These functions may be important in their antiviral and antitumour activities, possibly because of improved recognition of foreign antigens on virus-infected and tumour cells. Interferons appear to have dual effects in inflammation: ifn γ secreted in the vicinity of an inflammatory lesion appears to enhance responses such as the localised Schwartzman reaction to endotoxin. However, systemically administered ifn γ suppresses some aspects of inflammation, possibly acting as a feedback regulator of endotoxin responses (Heremans et al (1987), Billiau (1988)).

5.2.3. Effects of cytokines on gene expression.

Cytokines have multiple effects which extend beyond inflammation and immunity. Many of these appear to overlap; this is especially true of IL-1 and TNF. Exposure to IL-1 or TNF results in increased gene expression in a variety of cell types. The genes which are induced include those for the T-lymphocyte IL-2-, C3b- and EGF-receptors, cytokines including granulocyte-macrophage colony stimulating factor, interferons and IL-3, oncogenes such as c-fos and c-myc and extracellular matrix components. Other genes which are induced include collagenase, insulin and metallothionein (Dinarello (1988)). IL-1 and TNF also exert positive feedback regulation on their own expression. Some genes are suppressed by IL-1 and TNF: these include albumin, lipoprotein lipase and glucocorticoid-inducible phosphoenolpyruvate carboxykinase (Harrison and Campbell (1988)).

Interferons have profound effects on gene expression in many cell types: the family of interferon activated genes is thought to number 15 - 20 in most cells (Revel and Chebath (1986)). Some interferon-activated genes have been identified, purified and cloned: the existence of others has been deduced from interferon- induced changes in cellular functions or profiles on 2-dimensional SDS-PAGE analysis. Interferon-activated genes identified to date include (2',5') oligo-(A)- synthetase , which is thought to be involved in the regulation of cell growth and differentiation by interferons, the mouse Mx gene, which is involved in resistance to influenza virus, and members of the major histocompatibility complex, including HLA-A, B,

C, DRα and DRβ and β_2 microglobulin. Metallothionein, thymosin β4 and GTP/GDP binding protein genes are also induced by interferons, together with less well-understood genes such as the 1-8, 6-16 and 9-27 genes (Revel and Chebath (1986)). The roles of these genes in interferons' effects are the subject of intense study, especially because of the use of interferons in cancer therapy. Interferons also down-regulate the expression of some genes, obvious examples being viral genes which are suppressed in the "antiviral state" induced by interferons.

5.2.4. Potentiation and suppression of mouse liver P-450 isozymes during the acute phase response induced by bacterial endotoxin.

All of the above cytokines may be involved in the effects of inflammation on P-450 activity. Preliminary studies on the effects of E.coli endotoxin on P-450 expression in the mouse liver showed that treatment of male CBA mice with endotoxin had differential effects on hepatic P-450 concentration: a low dose (2μg/day injected intraperitoneally in 0.9% saline) did not affect total hepatic P-450 whereas the level was decreased following daily doses of 7.5μg or a single dose of 25μg. In mice treated concomitantly with endotoxin and PB or 3-MC, P-450 content was only reduced at the highest dose of endotoxin used. The effects of endotoxin on cytochrome b_5 concentrations followed a similar pattern to those on P-450 content, with some suppression occurring at the highest endotoxin dose (Table 5.2). In uninduced animals endotoxin had marginal effects on reductase content, but when administered together with PB low doses of endotoxin increased this activity (Figure 5.1a). The effects of endotoxin on cytochrome P-450-dependent monooxygenase activities was also studied (Figure 5.1 b-d). In control mice, increasing endotoxin doses generally caused a decrease in the metabolism of the three monooxygenase substrates tested (7-EC O-deethylase, EROD and benzphetamine), the exception being an elevation in 7-EC metabolism at the lowest endotoxin dose tested. The metabolism of all three substrates was strongly suppressed by the highest dose of endotoxin. In animals treated with PB, a different pattern of effects was observed, the metabolism of all three substrates being elevated at lower endotoxin doses. The most striking change observed was potentiation of 3-MC induction by low doses of endotoxin. Induction of EROD activity by 3-MC alone (10 fold) was increased to 24 fold in the presence of low doses of endotoxin.This effect was only observed at low endotoxin doses; at the highest dose of endotoxin suppression of EROD activity was observed compared with the effect of 3-MC alone. Potentiation of induction of P-450-dependent activities by

low doses of endotoxin was intriguing, since previous reports indicated only suppression of P-450-dependent activities. The mechanism of this effect was not clear; elevated reductase activity could have accounted for the potentiation of P-450-dependent activities in animals treated with PB plus low doses of endotoxin, but in animals treated with 3-MC plus endotoxin no increase in reductase activity occurred. It was important, therefore, to determine whether the increases in enzyme activity were accompanied by changes in P-450 protein expression; the first objective of the present work was to use Western blot analysis to discover whether this was the case. The results of this analysis are described below.

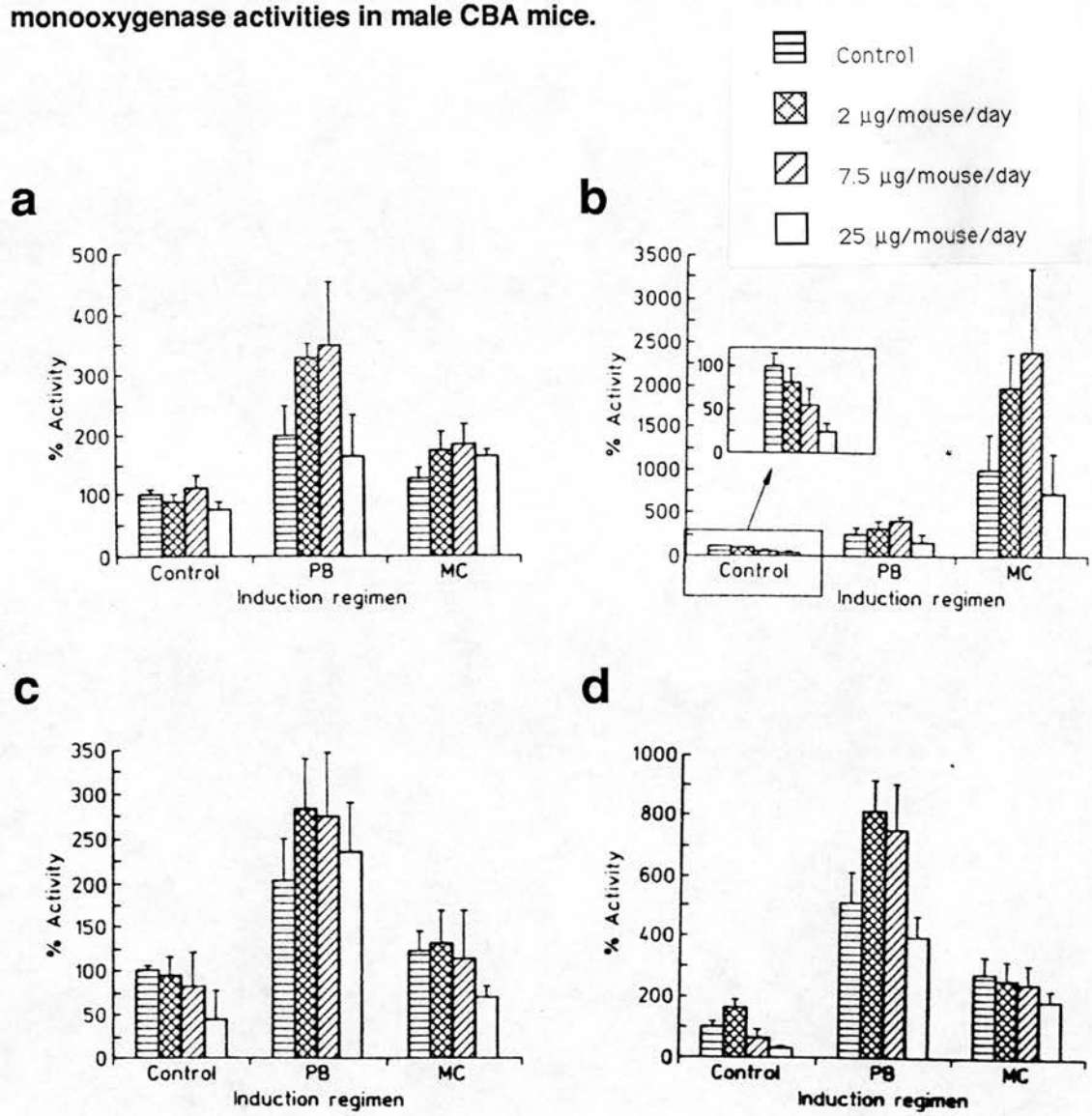
Table 5.2.

Effect of endotoxin on components of the cytochrome P-450-dependent monooxygenase system in mouse liver

Endotoxin dose	Cytochrome P-450* (nmol/mg protein)		Cytochrome b_5** nmol/mg protein)	
Control	0.59 ± 0.129	(100%)	0.18 ± 0.041	(100%)
2.0μg/day	0.61 ± 0.086	(103%)	0.17 ± 0.019	(94%)
7.5μg/day	0.42 ± 0.148	(71%)	0.16 ± 0.029	(89%)
25μg (x1)	0.36 ± 0.058	(61%)	0.10 ± 0.016	(56%)
PB (no endotoxin)	1.14 ± 0.357	(193%)	0.28 ± 0.026	(156%)
PB + 2.0μg/day	1.16 ± 0.335	(197%)	0.32 ± 0.063	(178%)
PB + 7.5μg/day	1.36 ± 0.221	(231%)	0.31 ± 0.050	(172%)
PB + 25μg (x1)	0.66 ± 0.112	(112%)	0.25 ± 0.054	(139%)
MC (no endotoxin)	0.88 ± 0.286	(149%)	0.31 ± 0.016	(172%)
MC + 2.0μg/day	1.02 ± 0.454	(173%)	0.26 ± 0.037	(144%)
MC + 7.5μg/day	0.59 ± 0.091	(100%)	0.20 ± 0.042	(111%)
MC + 25μg (x1)	0.54 ± 0.263	(92%)	0.21 ± 0.019	(117%)

*Mean ± S.D. for 6-9 mice. ** Mean ± S.D. for 3 mice.
Robert Lindsay and Lesley Rodger are thanked for performing these assays.

Figure 5.1.
Effect of endotoxin on hepatic NADPH -cytochrome P-450 reductase and monooxygenase activities in male CBA mice.



Male CBA mice were treated with PB or 3-MC and control animals were treated with saline. Endotoxin was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. The doses of endotoxin used were 0, 2, 7.5 or 25 µg/animal, equivalent to 0, 80, 300 or 1000µg/kg body weight. Liver microsomes were prepared as described in "Materials and Methods".
(a) NADPH-cytochrome P-450 reductase activity.
(b) 7-Ethoxyresorufin O-deethylation.
(c) Benzphetamine N-demethylation.
(d) 7-Ethoxycoumarin O-deethylation.
Activities are expressed as mean ± SD for 6 - 9 mice, except for (d) where 3 mice were used. All activities are expressed as a percentage of the activity of control (untreated) liver microsomes. The reductase actiovities were measured as nmol cytochrome c reduced/min/mg protein. Other activities were measured as nmol product produced/min/mg protein. Robert Lindsay and Lesley Rodger are thanked for performing these assays.

5.3. Effects of inflammatory mediators on mouse hepatic P-450 isozyme expression.

5.3.1. Western blot analysis of the effects of E. coli endotoxin.

Mouse hepatic samples from the study started by Dr.D.J. Adams and described in the introduction to this chapter were studied by Western blot analysis. Microsomal samples from three male mice were pooled, prepared for SDS-PAGE, and subjected to Western blot analysis loading 7.5μg of microsomal protein per track (Figures 5.2 - 5.4). Duplicate blots were also scanned using a Joyce-Loebl Chromoscan 3 densitometer in order to quantitate approximately the observed effects (Table 5.3).

Table 5.3.

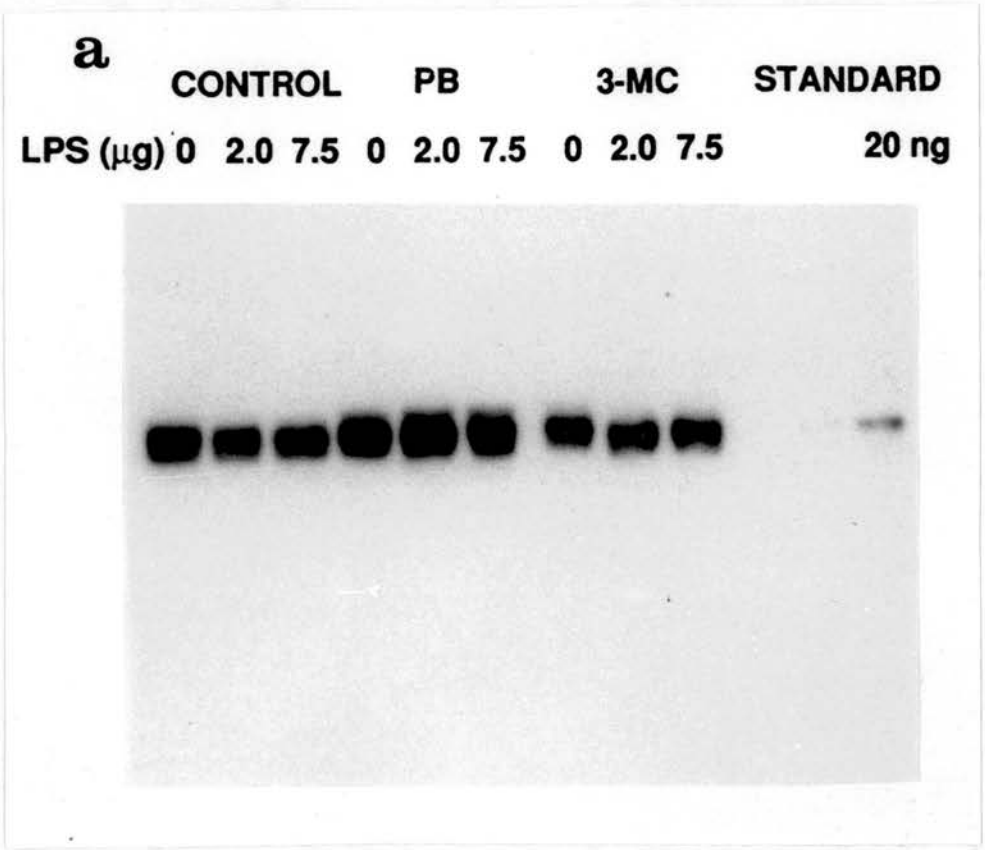
Use of densitometry to analyse Western blots on liver microsomes from mice treated with 0, 2 or 7.5μg/day E. coli endotoxin.

	Antiserum: αPB_1	αPB_{3a}	αMC_{1b}
C	100%	100%	100%
C + 2μg endotoxin	70%	68%	103%
C + 7.5μg endotoxin	88%	76%	93%
PB	116%	570%	166%
PB + 2μg endotoxin	113%	789%	191%
PB + 7.5μg endotoxin	111%	796%	211%
MC	83%	53%	635%
MC + 2μg endotoxin	75%	105%	702%
MC + 7.5μg endotoxin	73%	108%	749%

Relative band intensity was calculated as a percentage of control for pooled samples on duplicate blots; the average value is given.

Figure 5.2.

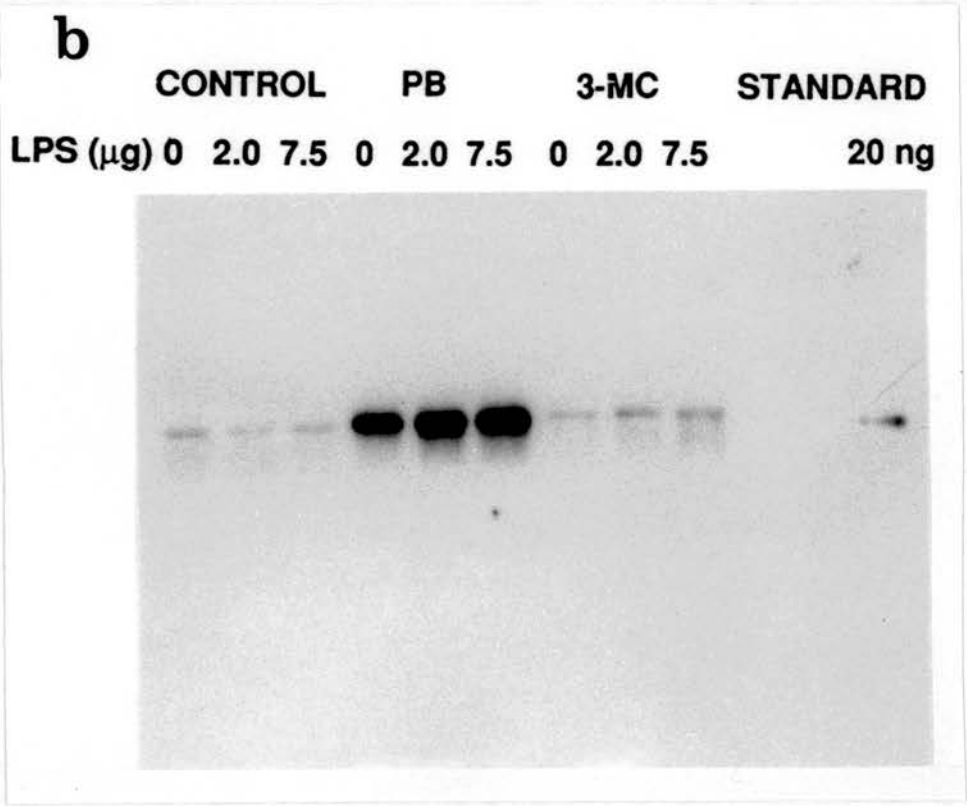
Effect of endotoxin on the hepatic expression of isozyme PB_1 in male CBA mice.



Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Endotoxin was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. The doses of endotoxin used were 0, 2 or 7.5 μg/animal, equivalent to 0, 80 or 300μg/kg. Liver microsomes were prepared as described. Microsomes from 3 mice were pooled together and analysed by Western blot analysis loading 7.5μg of microsomal protein per track.

Figure 5.3.

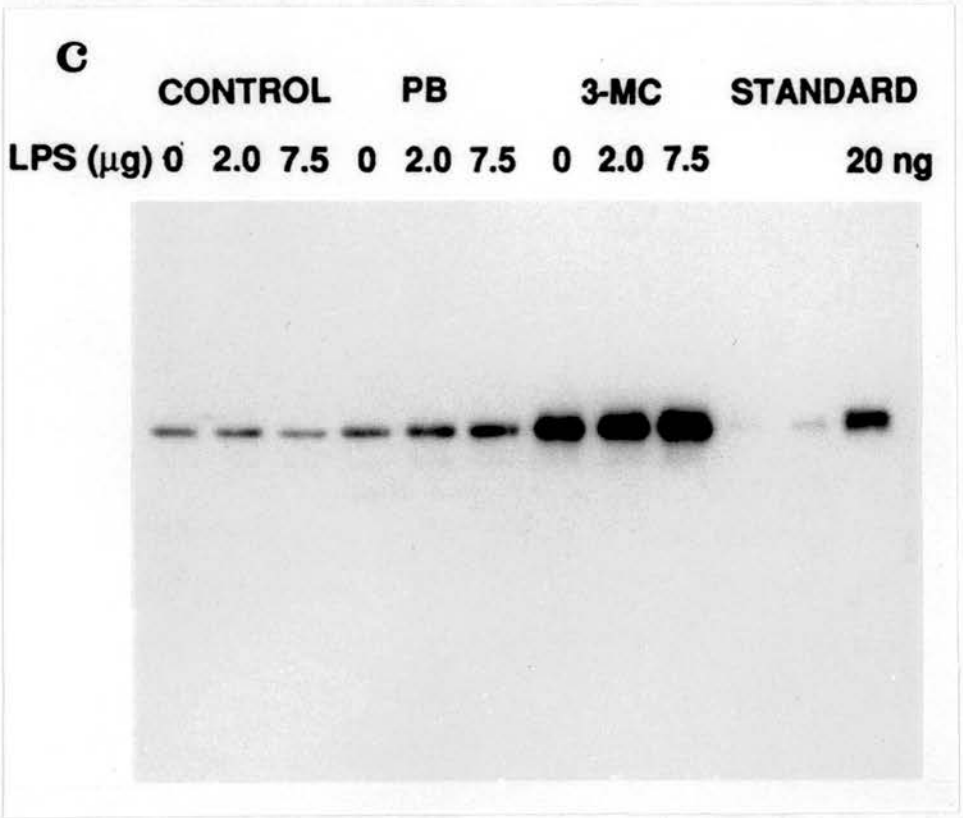
Effect of endotoxin on the hepatic expression of isozyme PB_{3a} in male CBA mice.



Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Endotoxin was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. The doses of endotoxin used were 0, 2 or 7.5 μg/animal, equivalent to 0, 80 or 300μg/kg. Liver microsomes were prepared as described. Microsomes from 3 mice were pooled together and analysed by Western blot analysis loading 7.5μg of microsomal protein per track.

Figure 5.4.

Effect of endotoxin on the hepatic expression of isozyme MC_{1b} in male CBA mice.



Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Endotoxin was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. The doses of endotoxin used were 0, 2 or 7.5 μg/animal, equivalent to 0, 80 or 300μg/kg. Liver microsomes were prepared as described. Microsomes from 3 mice were pooled together and analysed by Western blot analysis loading 7.5μg of microsomal protein per track.

Analysis of isozyme PB_1 in mouse liver microsomes showed that endotoxin slightly suppressed the expression of this isozyme in uninduced mice but had no suppressive effect in animals treated concomitantly with PB (Figure 5.2). Previous assays of EROD activity in these samples yielded similar results: this activity is thought to be mediated by PB_1 in uninduced liver (Wolf et al (1986)). As expected, the constitutive level of isozyme PB_{3a} was low in mouse liver; no significant effect of endotoxin was observed (Figure 5.3). However, induction of this isozyme by PB was potent- iated by treatment with 2 or 7.5μg/day endotoxin, the level in samples from mice treated with both PB and endotoxin being 1.4 times that in those treated with PB alone. This increase agreed closely with the elevated benzphetamine N-demethyase activity observed in these samples, and was similar to the results of 7-EC O-de- ethylase assays carried out previously. Concomitant treatment of mice with 3-MC and endotoxin caused a slight increase in hepatic microsomal MC_{1b} content compared with that in mice treated with 3-MC alone (Figure 5.4); however, the increase was not sufficient to account for the potentiation of 3-MC-induction of EROD activity by endotoxin, suggesting that another mechanism must be involved in this effect.

5.3.2. Effects of recombinant interferon α .

Another inflammatory mediator whose effect on P-450 expression was previously studied in this laboratory was ifn α (D.J. Adams, personal communication). Groups of three male CBA mice were treated intraperitoneally with PB (80mg/kg) or 3-MC (40mg/kg) for three days prior to sacrifice, with or without concomitant treatment with recombinant ifn α (25,000 u/mouse/day). Preliminary results indicated that P-450 level, reductase activity, 7-EC O-deethylation and benzphetamine N-demethylation were suppressed by interferon in uninduced mice; suppression of 7-EC O-deethylation was statistically significant ($p< 0.01$) (Table 5.4, Figure 5.5). In PB-induced mice, ifn α suppressed P-450-dependent activities; hepatic microsomal benzphetamine N-demethylation was significantly suppressed ($p<0.01$) in mice treated with PB plus ifn α compared with those treated with PB alone. Ifn α had no significant effect on the above parameters in 3-MC treated mice.

Table 5.4.

Effects of interferon α on mouse hepatic cytochromes P-450 and b_5.

	Cytochrome P-450 (nmol/mg protein)		Cytochrome b_5 (nmol/mg protein)	
Control	0.942 ± 0.093	(100%)	0.687 ± 0.024	(100%)
Control + ifn	0.736 ± 0.022	(78%)	0.641 ± 0.012	(93%)
PB	1.439 ± 0.076	(153%)	0.904 ± 0.030	(132%)
PB + ifn	0.954 ± 0.050*	(101%)	0.688 ± 0.010*	(100%)
3-MC	0.840 ± 0.108	(89%)	0.800 ± 0.023	(116%)
3-MC + ifn	0.892 ± 0.036	(95%)	0.735 ± 0.018	(107%)

* Significantly different from non-interferon treated sample $p<0.01$.
Lesley Rodger is thanked for performing these assays.

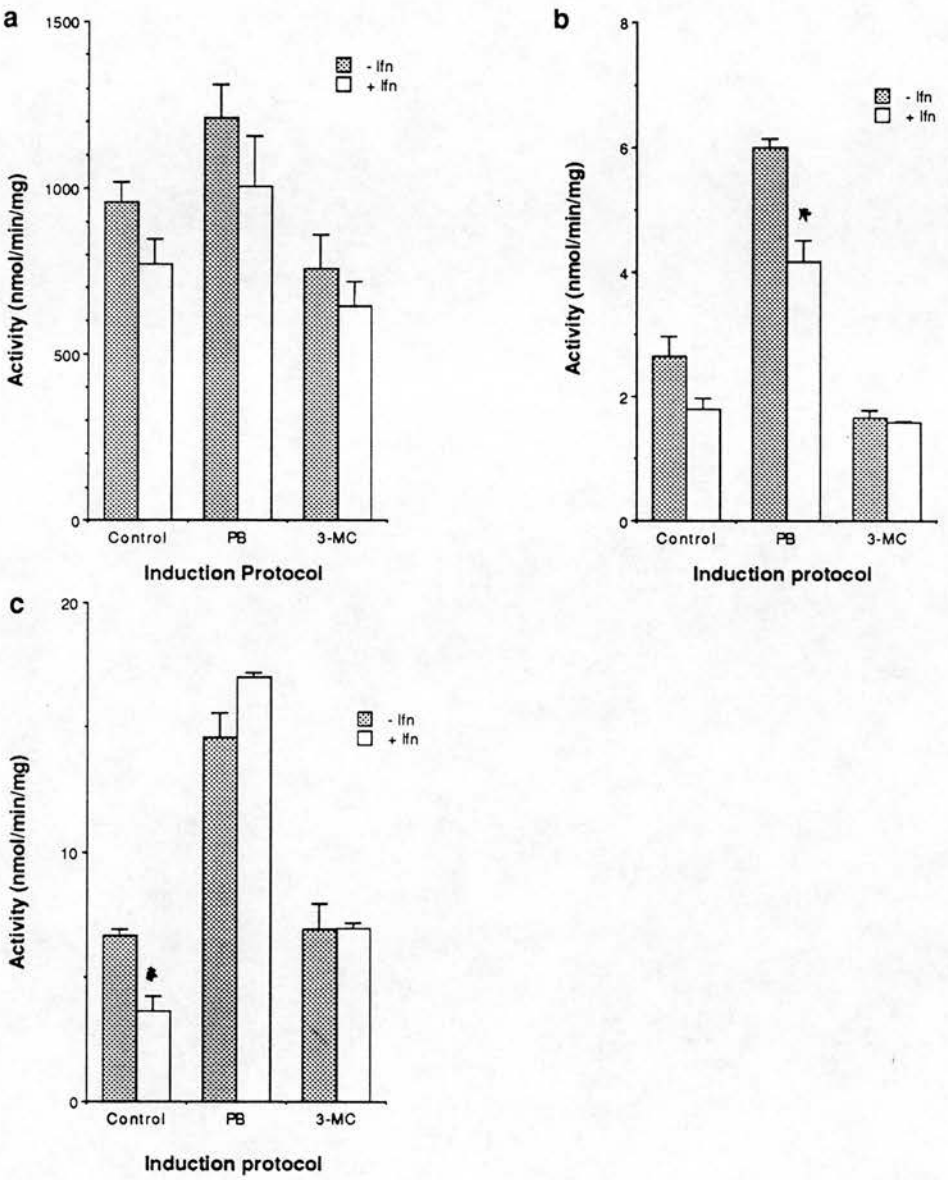
In order to confirm and extend these data, the effect of ifn α on metabolism of 7-PR and 7-BR was determined (Figure 5.6 b,c). Ifn α significantly suppressed constitutive and PB-induced PROD activity ($p<0.05$); its effect on BROD activity was similar, but the differences observed were not statistically significant. These results agreed with previous studies on benzphetamine N-demethylation in these samples. The results of previous work on EROD activity in these samples were in marked contrast to the above findings (L. Rodger, personal communication). Ifn α significantly suppressed EROD activity in uninduced mice ($p<0.05$) but in 3-MC treated mice induction was potentiated by ifn α to about 1.6 times that in mice treated with 3-MC alone (Figure 5.6a). This potentiation was similar to that observed after treatment of mice with endotoxin plus 3-MC, though the effect of ifn α was less marked than that of endotoxin.

Western blot analyses were performed to establish whether the observed changes in

P-450-dependent enzyme activities after ifn α treatment were reflected at the level of P-450 isozyme expression. Two PB-inducible isozymes (PB_1 and PB_{3a}) and two 3-MC-inducible isozymes (MC_{1a} and MC_{1b}) were studied. Hepatic microsomes from three mice were pooled together and subjected to Western blot analysis loading 7.5μg protein per track. The results indicated that PB_1 expression was slightly suppressed by ifn α irrespective of whether the animals were treated with PB or 3-MC (Figure 5.7a). Expression of PB_{3a} in uninduced or 3-MC-treated animals was too low to draw conclusions about the effect of ifn α on constitutive expression of this isozyme, but in animals treated with both PB and ifn α slight suppression of the induced level of PB_{3a} occurred (Figure 5.7b). Two bands on this blot were labelled by the anti-serum raised against rat PB_{3a}: these may represent mouse isozymes equivalent to the two highly homologous proteins PB_{3a} and PB_{3b} (Ryan et al (1982), Suwa et al (1985)). In the case of the 3-MC-inducible isozymes, ifn α had no detectable effect on the induced level of MC_{1a} or MC_{1b}, but the expression of MC_{1a} in uninduced and PB-treated mice was slightly suppressed by ifn α (Figure 5.8a). Ifn α had little, if any, effect on the expression of MC_{1b} in either induced or uninduced animals (Figure 5.8b). In order to quantify these results and to discover whether the observed effects were significant, further Western blot analyses were carried out using individual mouse liver microsomes. Microsomes from the three mice in each of the groups to be compared were analysed on the same Western blot and the autoradiographs scanned using a Joyce-Loebl Chromoscan 3 densitometer. Band intensity was calculated as a percentage of that of control band 1 and the mean percentage intensity (± standard deviation) calculated. The results of this analysis are shown in Table 5.5. This analysis showed that the suppression of the constitutively expressed isozymes PB_1 and MC_{1a} in uninduced mouse liver by ifn α was statistically significant, but that the slight effects of ifn α on induced protein levels in these samples, and the effects on the two inducible isozymes were not significant. The fact that significant suppression of these isozymes was not observed in this analysis, whereas on the Western blots using pooled samples suppression of isozyme PB_{3a} appeared to have occurred, emphasises the difficulty of drawing firm conclusions from Western blots when the observed changes in protein expression are very small.

Figure 5.5.

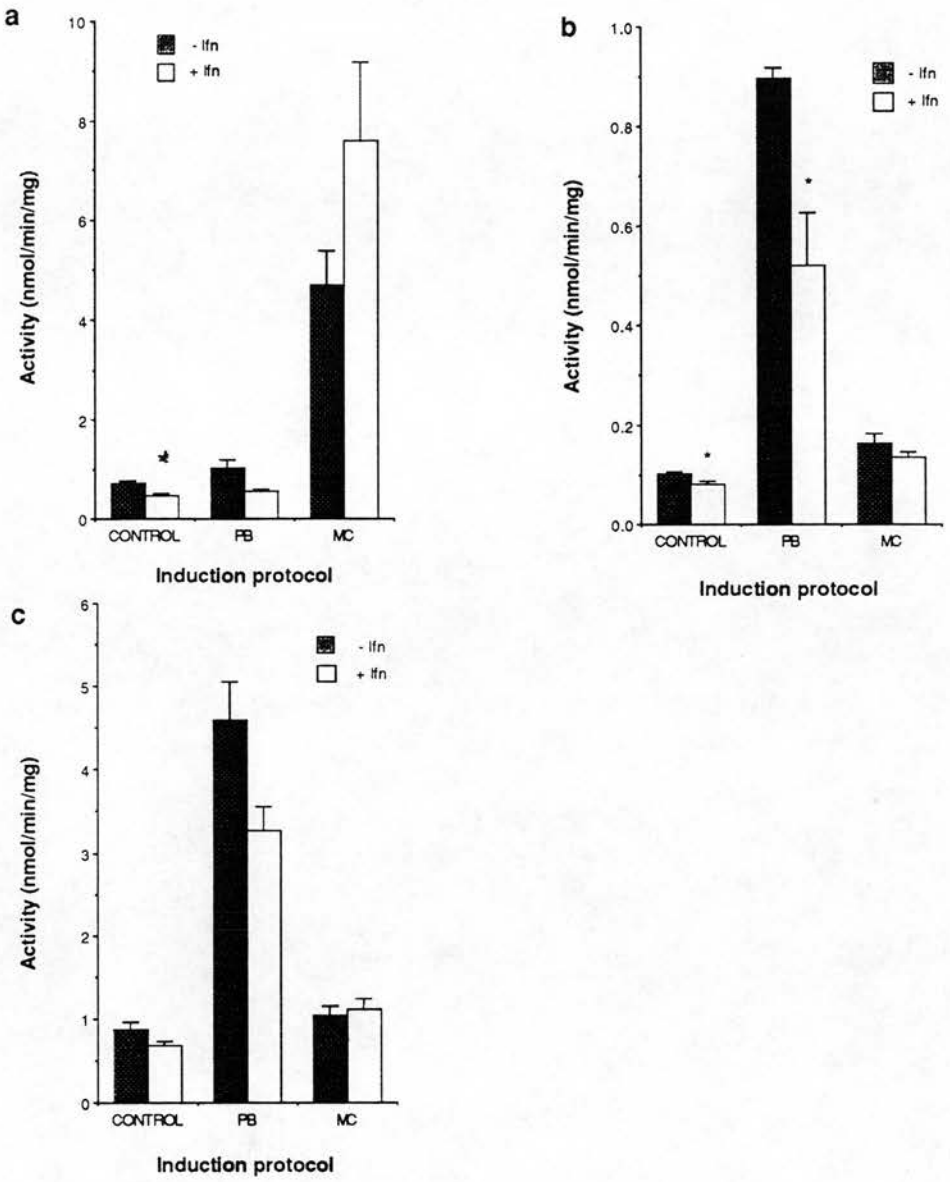
Effect of recombinant interferon α on hepatic NADPH-cytochrome P-450 reductase and monooxygenase activities in male CBA mice.



Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Recombinant ifn α (25,000 units/animal) was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. Liver microsomes were prepared as described. The figure shows mean activity ± SEM for individual hepatic microsomal samples from 3 mice. Lesley Rodger is thanked for performing these assays.
(a) NADPH-cytochrome P-450 reductase activity (nmol cytochrome c reduced/min/mg protein).
(b) Benzphetamine N-demethylation (nmol product produced/min/mg protein).
(c) 7-Ethoxycoumarin O-deethylation (nmol product produced/min/mg protein).

Figure 5.6.

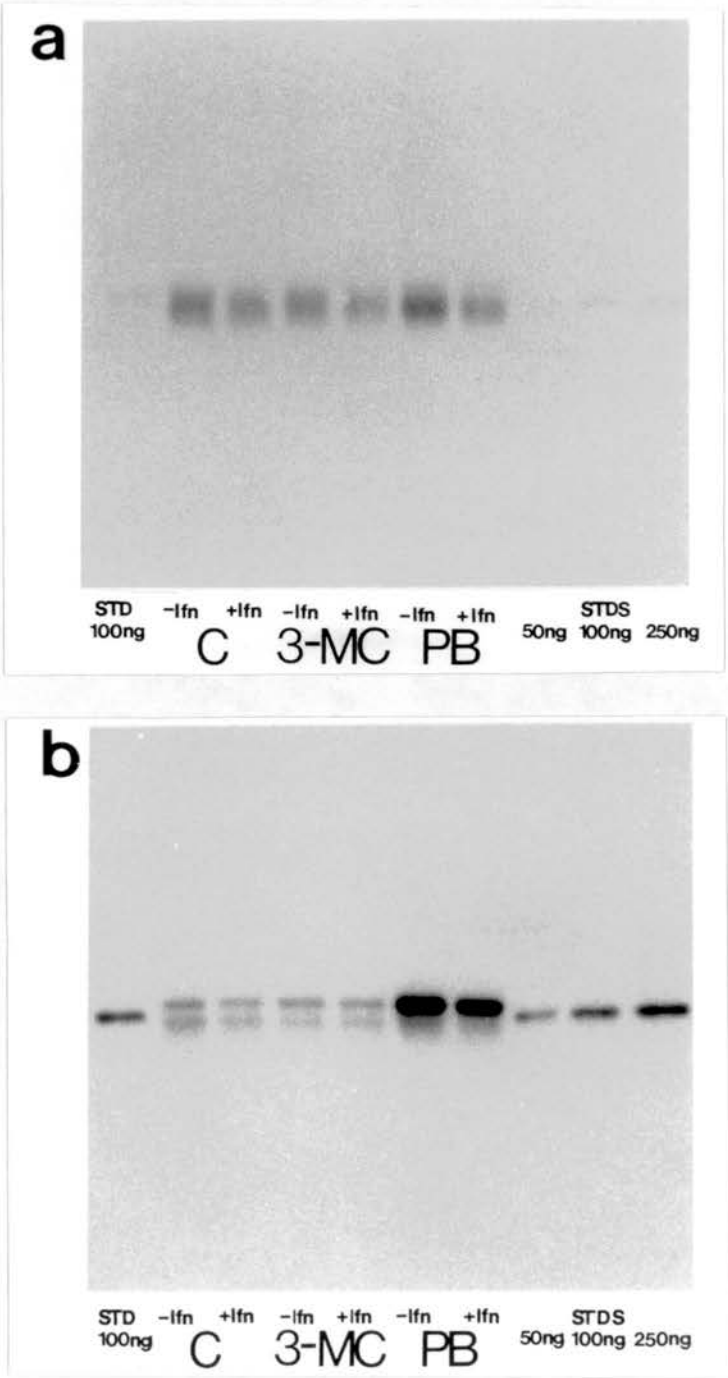
Effect of recombinant interferon α on hepatic microsomal alkoxyresorufin metabolism in male CBA mice.



Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Recombinant ifn α (25,000 units/animal) was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. Liver microsomes were prepared and alkoxyresorufin assays carried out by the direct method, as described. The figure shows mean activity ± SEM for individual hepatic microsomal samples from 3 mice.
(a) EROD activity (nmol/min/mg microsomal protein).
Lesley Rodger is thanked for performing this assay.
(b) PROD activity (nmol/min/mg microsomal protein).
(c) BROD activity (nmol/min/mg microsomal protein).
*Significantly different from non-interferon treated sample $p<0.05$.

Figure 5.7.

Effect of recombinant interferon α on the hepatic expression of isozymes PB_1 and PB_{3a} in male CBA mice.



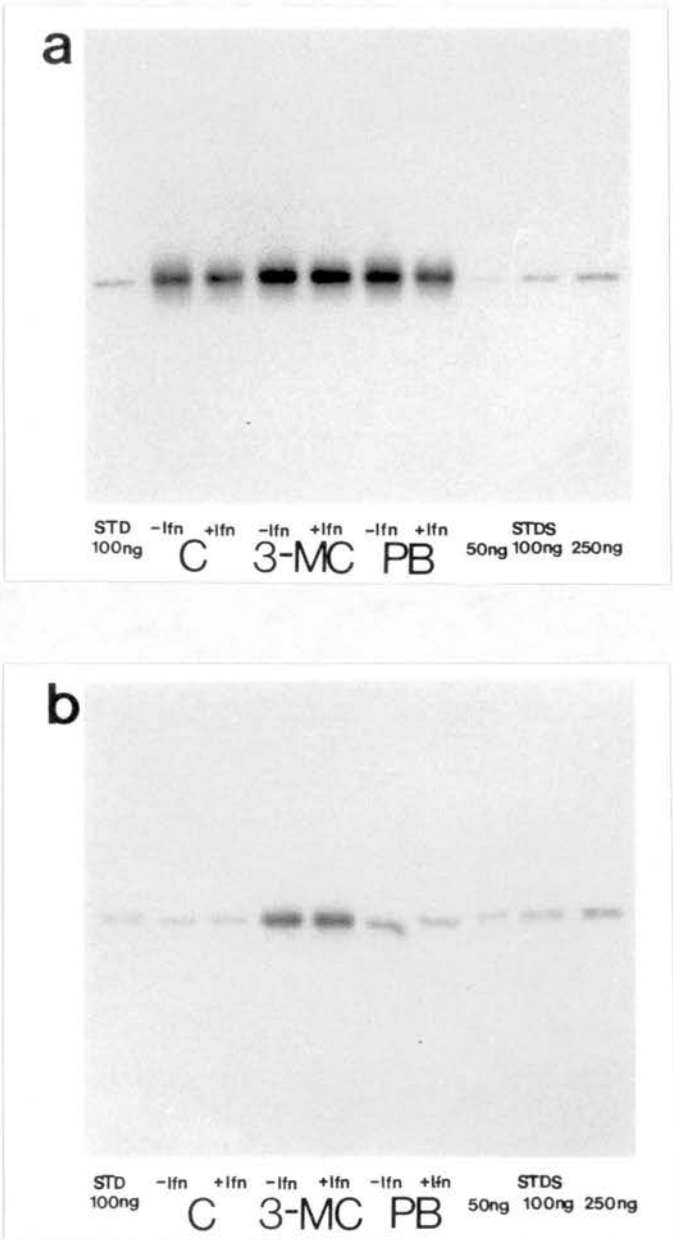
Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Recombinant ifn α (25,000 units/animal) was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. Liver microsomes were prepared as described. Microsomes from 3 mice were pooled together and analysed by Western blot analysis loading 7.5μg of protein per track.

(a) Expression of isozyme PB_1.

(b) Expression of isozyme PB_{3a}.

Figure 5.8.

Effect of recombinant interferon α on the hepatic expression of isozymes MC_{1a} and MC_{1b} in male CBA mice.



Male CBA mice were treated with PB (80mg/kg) or 3-MC (40mg/kg) and control animals were treated with saline. Recombinant ifn α (25,000 units/animal) was injected intraperitoneally immediately after administration of PB or 3-MC on the three days prior to sacrifice. Liver microsomes were prepared as described. Microsomes from 3 mice were pooled together and analysed by Western blot analysis loading 7.5μg of protein per track.

(a) Expression of isozyme MC_{1a}.

(b) Expression of isozyme MC_{1b}.

Table 5.5.

Use of densitometry to analyse the effects of interferon α on P-450 isozyme expression in mouse liver.

	Antiserum:			
	αPB_1	αPB_{3a}	αMC_{1a}	αMC_{1b}
C	101 ± 4	124 ± 12	105 ± 5	121 ± 11
C + ifn α	71 ± 1*	132 ± 14	61 ± 2*	108 ± 25
PB	97 ± 4	970 ± 98	N B	N B
PB + ifn α	94 ± 4	886 ± 88	N B	N B
MC	N B	N B	100 ± 6	586 ± 54
MC + ifn α	N B	N B	82 ± 5	517 ± 41

Relative band intensity was calculated as a percentage of that of the first control sample. Values are mean ± standard deviation for three individual mouse liver microsomal samples run on the same blot.
* Significantly different from non-interferon treated sample $p < 0.005$.
N D - Not blotted.

In conclusion, recombinant ifn α significantly suppressed constitutive P-450 levels in mouse liver, both at the level of metabolic activity and expression of specific isozymes. The effect of ifn α on inducible P-450s was less clear; some inducible activities (benzphetamine N-demethylation, PROD) were suppressed by ifn α, but EROD induction was potentiated by ifn α. No significant effect of ifn α on inducible isozyme expression was detected by Western blot analysis. Comparison of these effects with those of endotoxin showed that some effects of ifn α, including potentiation of 3-MC induction of EROD, were similar to those of endotoxin. However, ifn α and endotoxin had opposing effects on PB-induced benzphetamine N-demethylation.

5.4. Effects of inflammatory mediators on 7-ethoxyresorufin metabolism in NCI H322 cells.

In an attempt to dissect the complex effects of inflammatory mediators on P-450 expression, it was decided to adopt a cell culture model in which the effects of several mediators could be studied. In view of possible links between inflammation, P-450 induction and cancer in the human lung, the human lung tumour-derived cell line NCI H322 was chosen for these studies; however, it should be noted that P-450 regulation by inflammatory mediators in the lung cell line may be different from that in the liver.

Recombinant ifns α, β and γ and TNF were supplied by Dr. F. Balkwill, Imperial Cancer Research Fund, 44, Lincoln's Inn Fields, London WC2A 3PX; IL-1 was kindly donated by Dr. F. DiGiovine, Northern General Hospital, Edinburgh. The first series of experiments on NCI H322 cells was designed to test the direct effect of endotoxin on the metabolism of 7-ER in NCI H322 cells and to compare this with the effects of TNF, IL-1 and Dex, that is, two mediators of endotoxin effects and a synthetic glucocorticoid hormone analogue. In these experiments NCI H322 cells were grown to confluency in 25cm^2 flasks, then treated for 24 hours with either BA (5µg/ml) or DMSO (control), with or without concomitant endotoxin, TNF, IL-1 or Dex treatment. A similar series of experiments was performed using recombinant human ifns α, β and γ.

5.4.1. Effects of E. coli endotoxin and mediators of its activity.

Endotoxin had no significant effect on EROD activity in NCI H322 cells (Figure 5.9). In uninduced cells, slight suppression of EROD occurred, the activity of the endotoxin treated cells being 87.6% of control. Endotoxin had no effect on BA-induced EROD in NCI H322 cells; the activity of cells treated with BA plus endotoxin was 104.7% of that of cells treated with BA alone. Similarly, TNF had very little effect on metabolism of 7-ER in this cell line, the activity of TNF-treated cells being 91.2% of the control activity, whilst the activity of cells treated with BA plus TNF was the same as that of cells treated with BA alone (99.2%) (Figure 5.10). The effect of IL-1 was slightly more marked than those of endotoxin or TNF: IL-1 suppressed constitutive EROD activity to 82.6% of control ($p<0.05$) (Figure 5.11). Similarly, IL-1 caused about 10% suppression of BA-induced activity, to 89.9% of that in cells treated with BA alone. However, this suppression was not statistically significant.

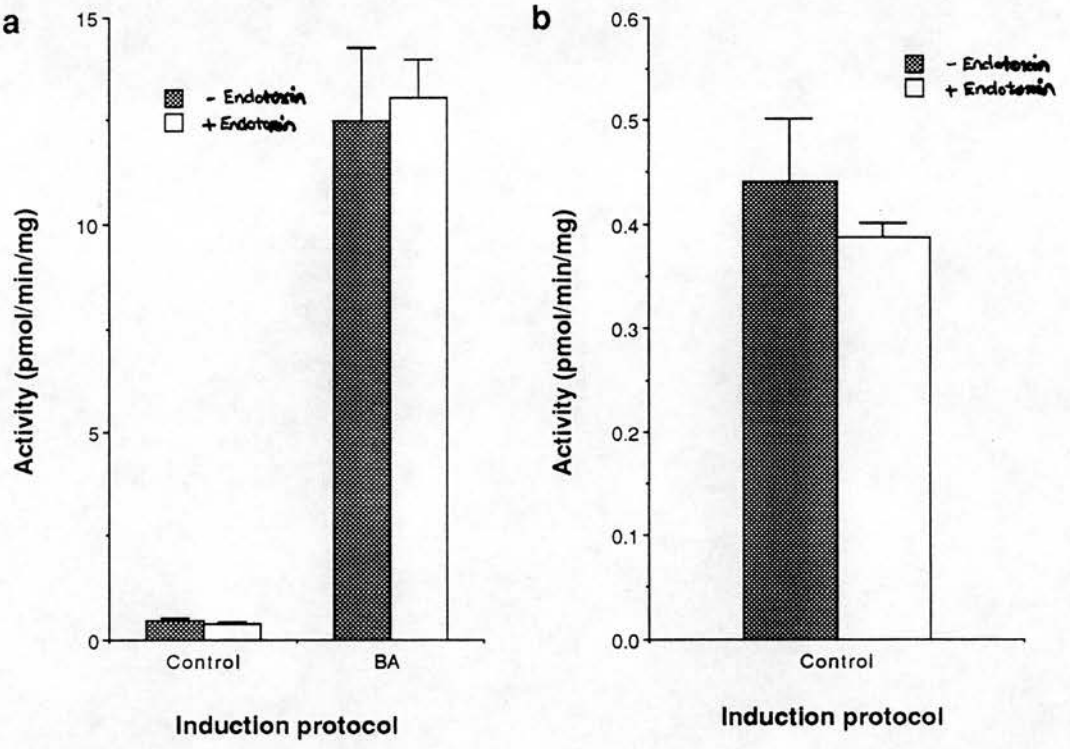
The synthetic glucocorticoid, Dex, had more marked effects on EROD activity in NCI H322 cells; like the other three agents, Dex caused some suppression of constitutive EROD activity to 79.2% of control (Figure 5.12). However, Dex reduced the BA-induced EROD activity of the cells by almost half, to 52.8% of that of BA-treated cells ($p<0.05$).

5.4.2. Effects of interferons α, β and γ.

The type I interferons (ifns α and β) had slight effects on EROD activities in NCI H322 cells: ifn α had no effect on constitutive 7-ER metabolism, the activity of ifn α-treated cells being 102.2% of control, although this interferon did suppress BA-induced EROD activity by about 20%, to 78.3% of that of cells treated with BA alone (Figure 5.13). Ifn β caused a slight decrease in both constitutive and induced activities, to 85.3% and 81.9%, respectively, of the relevant control activities (Figure 5.14). In contrast, Type II interferon (ifn γ) had marked effects; the constitutive EROD activity of NCI H322 cells was reduced to 47.5% of control by treatment with ifn γ ($p<0.05$) and BA-induced activity was suppressed even more dramatically, cells treated with BA together with ifn γ having only 27.7% of the activity of cells treated with BA alone ($p<0.01$) (Figure 5.15). The effects of the inflammatory agents on EROD activity in NCI H322 cells are summarised below (Table 5.6). These results suggest that several cytokines suppress EROD activity in NCI H322 cells, and significant effects are also caused by glucocorticoid hormones, represented by the synthetic glucocorticoid Dex. The cytokine with the most marked influence on EROD activity in NCI H322 cells was ifn γ, though the other interferons did suppress this activity a little. No potentiation of either constitutive or BA-induced EROD activity by these agents was detected.

Figure 5.9.

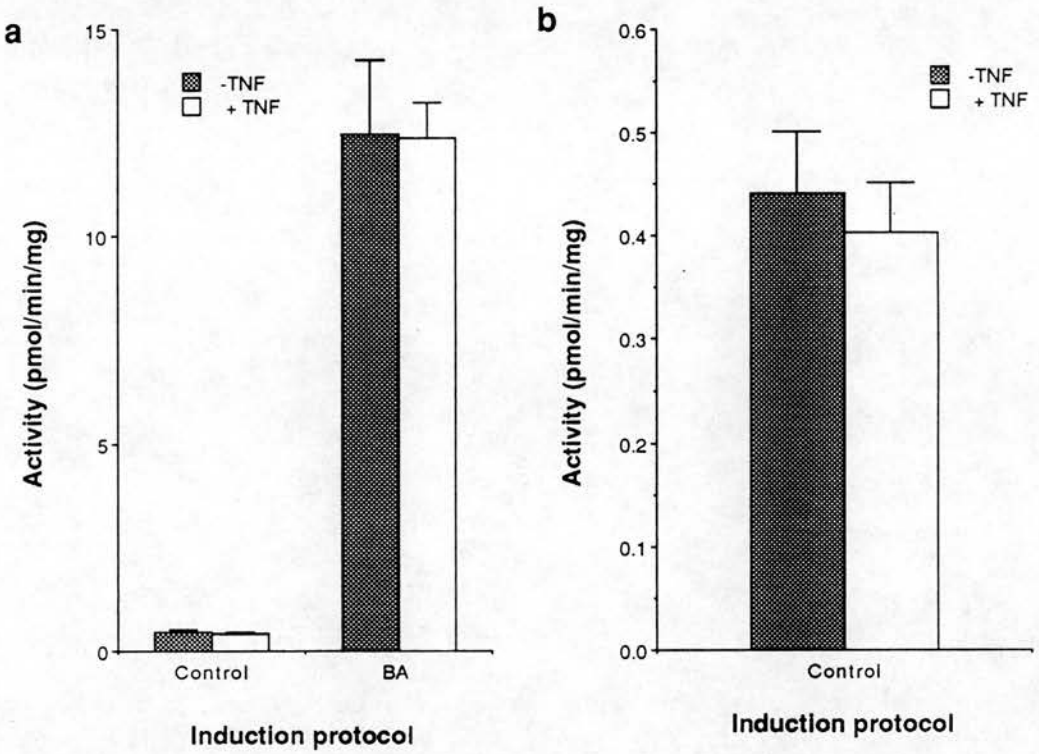
Effects of E. coli endotoxin on constitutive and BA-induced EROD activity in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5µg/ml) or DMSO (control) with or without E. coli endotoxin (1µg/ml).The cells were harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.
(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of endotoxin.
(b) EROD activity of uninduced cells in the presence and absence of endotoxin. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of endotoxin on constitutive EROD activity.

Figure 5.10.

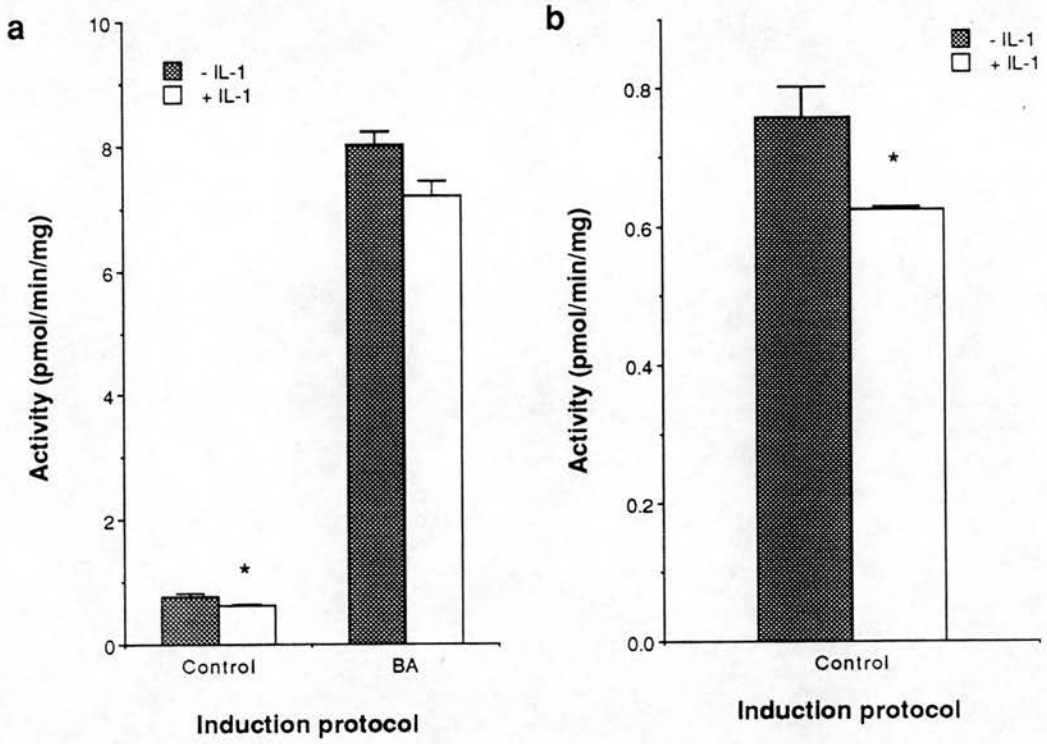
Effects of recombinant human TNF on constitutive and BA-induced EROD activity in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5µg/ml) or DMSO (control) with or without TNF (500ng/ml).The cells were harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.
(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of TNF.
(b) EROD activity of uninduced cells in the presence and absence of TNF. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of TNF on constitutive EROD activity.

Figure 5.11.

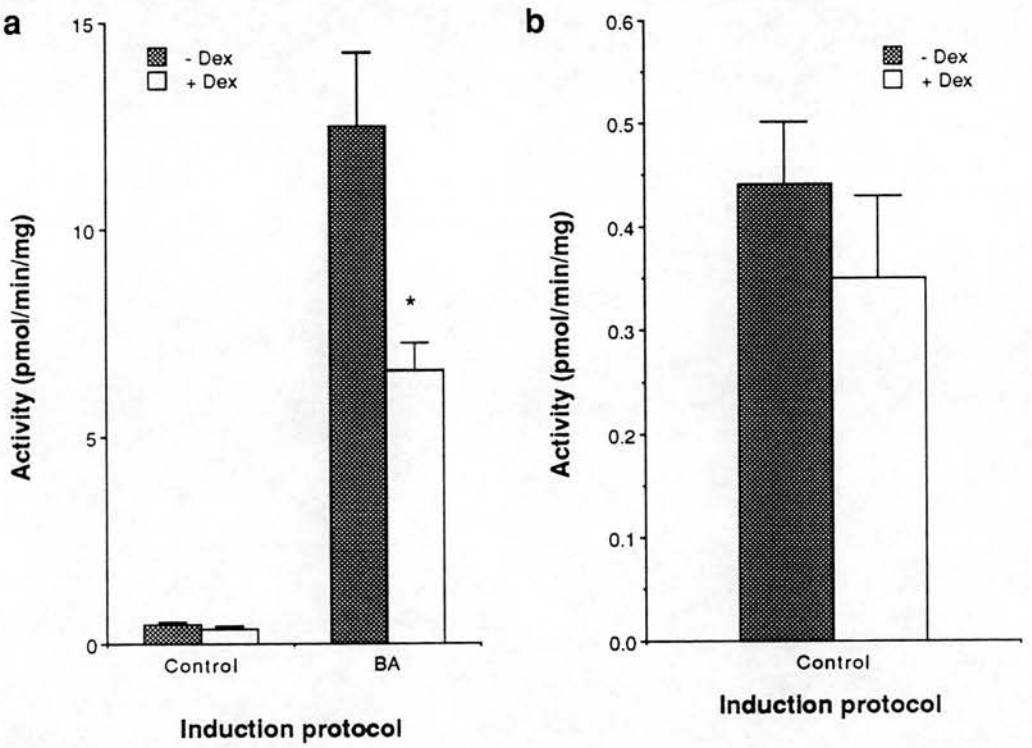
Effects of recombinant human IL-1β on constitutive and BA-induced EROD activity in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5μg/ml) or DMSO (control) with or without IL-1β (1ng/ml).The cells were harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.
(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of IL-1β.
(b) EROD activity of uninduced cells in the presence and absence of IL-1β. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of IL-1β on constitutive EROD activity.
* Significantly different from non-IL-1 treated sample, $p<0.05$.

Figure 5.12.

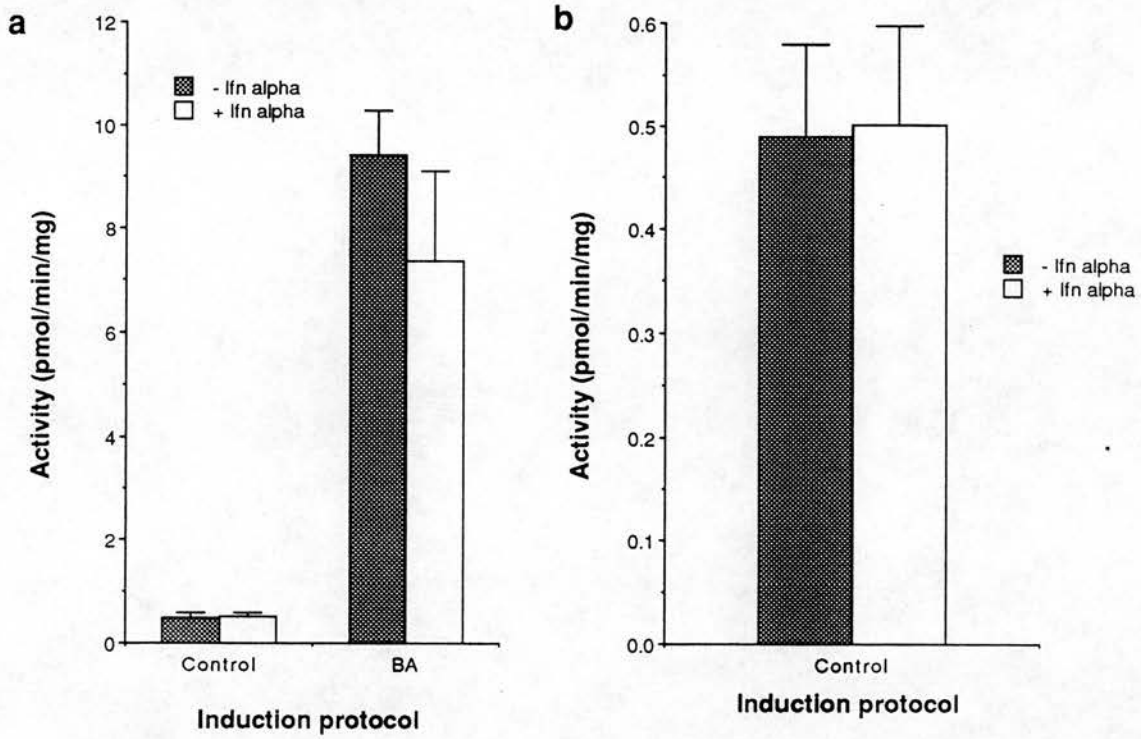
Effects of dexamethasone on constitutive and BA-induced EROD activity in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5µg/ml) or DMSO (control) with or without Dex (10µg/ml).The cells were harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.
(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of Dex.
(b) EROD activity of uninduced cells in the presence and absence of Dex. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of Dex on constitutive EROD activity.
* Significantly different from non-Dex treated sample, $p<0.05$.

Figure 5.13.

Effects of recombinant human interferon α on constitutive and BA-induced EROD activity in NCI H322 cells.



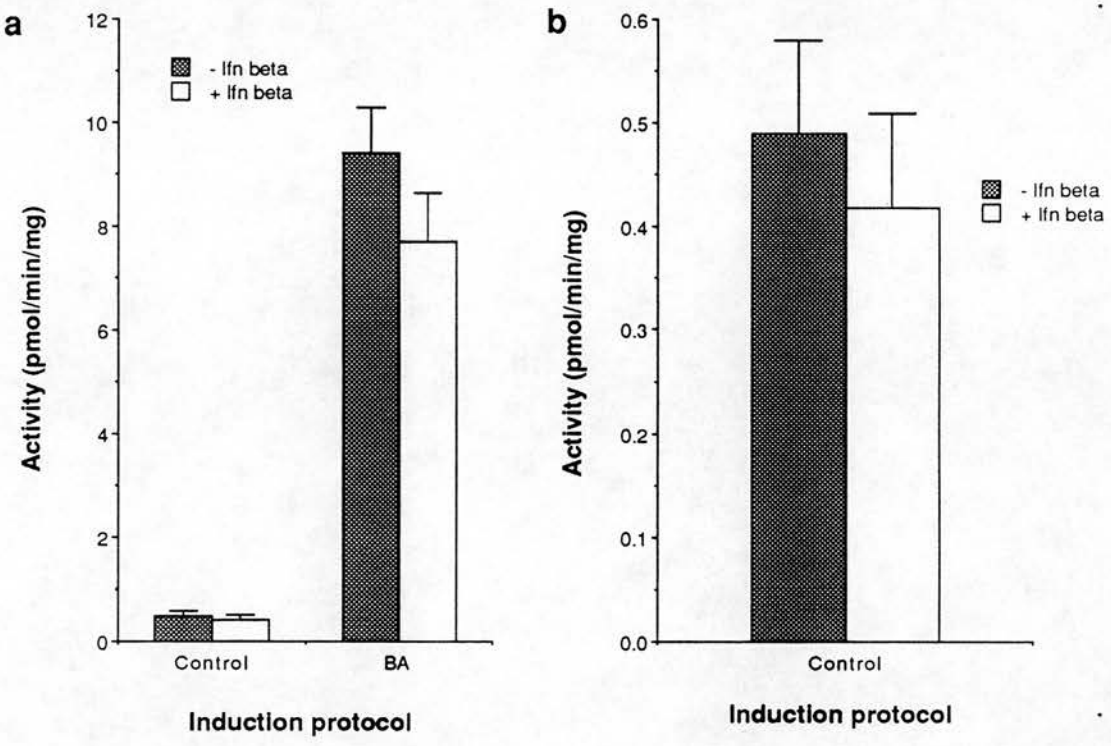
NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5μg/ml) or DMSO (control) with or without ifn α (1000u/ml).The cells were harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.

(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of ifn α.

(b) EROD activity of uninduced cells in the presence and absence of ifn α. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of ifn α on constitutive EROD activity.

Figure 5.14.

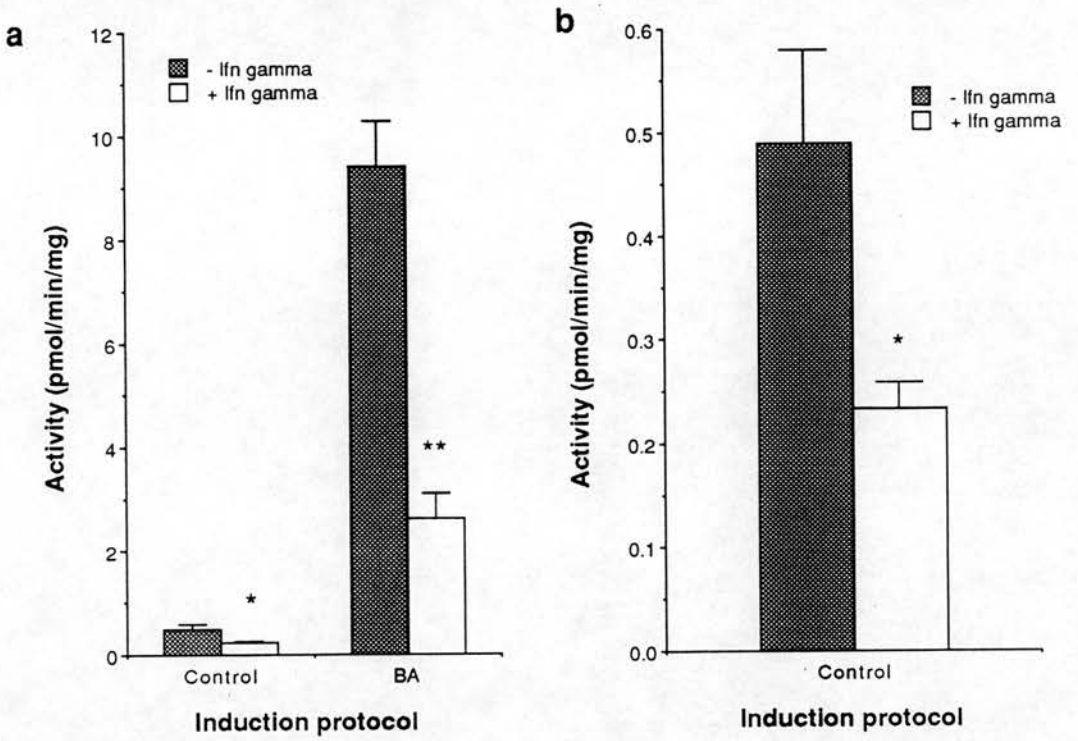
Effects of recombinant human interferon β on constitutive and BA-induced EROD activity in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5μg/ml) or DMSO (control) with or without ifn β (1000u/ml).The cells were harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.
(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of ifn β.
(b) EROD activity of uninduced cells in the presence and absence of ifn β. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of ifn β on constitutive EROD activity.

Figure 5.15.

Effects of recombinant human interferon γ on constitutive and BA-induced EROD activity in NCI H322 cells.



NCI H322 cells were grown to confluency in tissue culture flasks and treated for 24 hours with BA (5μg/ml) or DMSO (control) with or without ifn γ (1000u/ml).The cells weré harvested, sonicated and frozen at -40°C. When required, samples were diluted to approximately 1mg/ml and their alkoxyresorufin activities measured as described. The above figures show mean activity ± SEM for 3 samples.

(a) EROD activity in control and BA-treated NCI H322 cells induced in the presence and absence of ifn γ.

(b) EROD activity of uninduced cells in the presence and absence of ifn γ. This figure shows the same data as in (a) but on a larger scale to emphasise the effect of ifn γ on constitutive EROD activity.

* Significantly different from non-ifn γ treated sample $p < 0.05$.

** Significantly different from non-ifn γ treated sample $p < 0.01$.

Table 5.6.

Effects of inflammatory agents on 7-ethoxyresorufin metabolism in NCI H322 cells.

	% of control activity: Uninduced cells	BA-treated cells
Inflammatory agent:		
Endotoxin	87.6%	104.7%
TNF	91.2%	99.2%
IL-1	82.6% *	89.9%
Synthetic glucocorticoid:		
Dex	79.2%	52.8% *
Interferon:		
Ifn α	102.2%	78.3%
Ifn β	85.3%	81.9%
Ifn γ	47.5% *	27.7% **

NCI H322 cells were treated with BA (5μg/ml) or DMSO (control) with or without inflammatory mediators. The results represent the EROD activity present in inflammatory agent-treated cells as a percentage of that of relevant control cells.
* Significantly different from relevant control $p<0.05$.
** Significantly different from relevant control $p<0.01$.

5.4.3. Toxicity of inflammatory mediators.

One possible reason for the observed suppression of EROD activity in NCI H322 cells by inflammatory agents was exertion of toxic effects on the cells. To test this hypothesis, the toxicity of each of the agents used was tested using the MTT assay over a concentration range up to ten times that used in the induction experiments. The results of this analysis showed that endotoxin and IL-1 were non-toxic to this

cell line at concentrations up to ten times those used in induction experiments (Figures 5.16 and 5.17). The agent having the most marked toxicity was TNF, which caused approximately 60% reduction in final OD at all concentrations above 300ng/ml. All three interferons had very similar toxicity, having fairly shallow curves in which about 20% cell death occurred at the concentration used previously, increasing to 40% at 10,000u/ml. These results are summarised and compared with the suppression of constitutive EROD activity in Table 5.7.

Table 5.7.

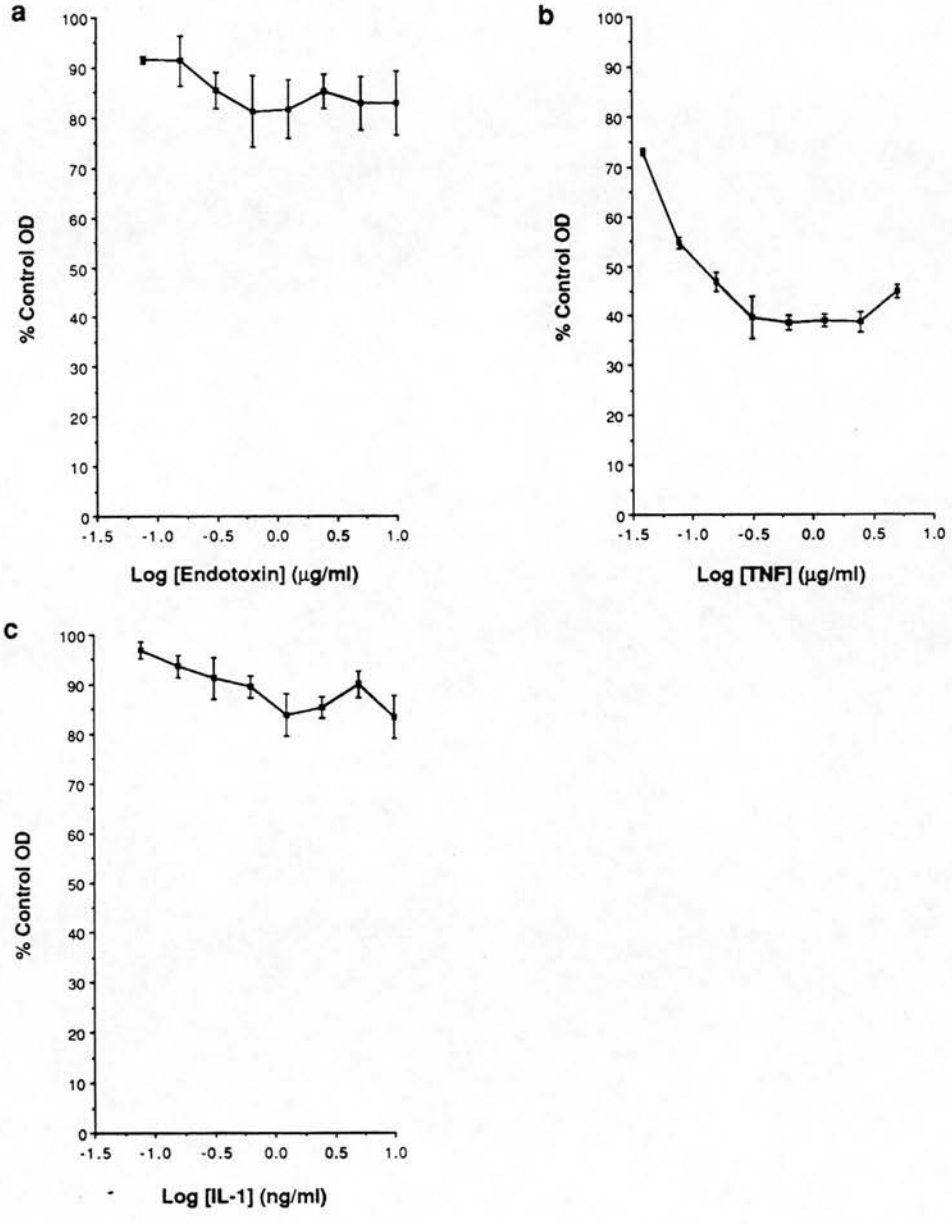
Comparison of the effects of inflammatory agents on EROD activity and cell growth in NCI H322 cells.

	% of control level: (a) EROD activity	(b) Cell growth
Inflammatory agent:		
Endotoxin	87.6%	81.0%
TNF	91.2%	40.0%
IL-1	82.6%	86.0%
Synthetic glucocorticoid:		
Dex	79.2%	~100% $
Interferon:		
Ifn α	102.2%	76.0%
Ifn β	85.3%	75.0%
Ifn γ	47.5%	86.0%

The results show (a) EROD activity of cells treated with inflammatory agent as a percentage of control activity (b) OD of cells treated with the concentration of inflammatory agent as a percentage of that of control cells in the MTT assay.
$ See Figure 4.11.

Figure 5.16.

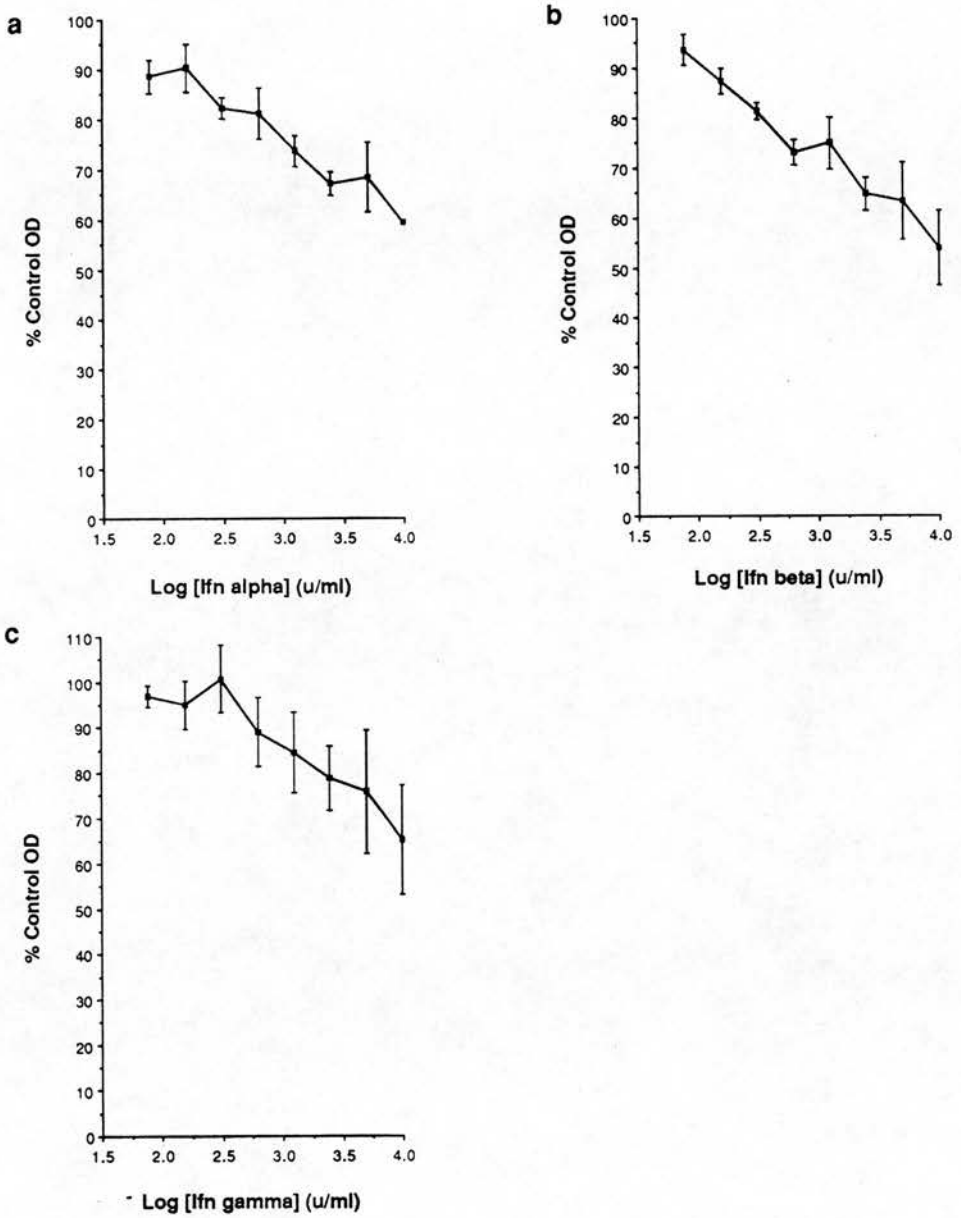
Toxicity of E. coli endotoxin, recombinant human TNF and recombinant human IL-1β towards NCI H322 cells.



MTT assays were set up as described previously, plating out 2500 NCI H322 cells per well in a volume of 180μl. The cells were allowed to adhere overnight and then 20μl of a 10x concentrated stock of endotoxin, TNF or IL-1β in serum-free medium was added to give final concentrations in a range up to 10 times those used in the induction experiments (Figures 5.7 - 5.9). The plates were incubated at 37°C for 5 more days then MTT was added and the plates developed as described.

Figure 5.17.

Toxicity of recombinant human interferons α, β and γ towards NCI H322 cells.



MTT assays were set up as described previously, plating out 2500 NCI H322 cells per well in a volume of 180µl. The cells were allowed to adhere overnight and then 20µl of a 10x concentrated stock of ifn α, β or γ in serum-free medium was added to give final concentrations in the range 78 - 10,000 u/ml (ie. up to 10 times the concentrations used in the induction experiments (Figures 5.11 - 5.13)). The plates were incubated at 37°C for 5 more days then MTT was added and the plates developed as described.

There was no correlation between toxicity and suppression of EROD activity by inflammatory agents, although some of the minor effects observed, such as the suppression of constitutive EROD activity by IL-1, could have been due to cell death. The two agents which severely suppressed EROD activity (Dex and ifn γ) were relatively non-toxic at the concentrations used, whereas the most toxic cytokine (TNF) did not cause a corresponding loss of EROD activity. In interpreting these results it should be noted that the MTT assay requires that cells be in logarithmic growth whereas the induction experiments were carried out using confluent cells. In induction experiments, no indications of loss of viability (such as cells detaching from the culture surface) were observed. These data indicate that toxicity is not the reason for suppression of EROD activity by inflammatory agents in NCI H322 cells.

5.5. Discussion.

In this chapter experiments were carried out to assess the effects of inflammatory mediators on P-450 expression in two systems, the CBA mouse liver and the human lung tumour-derived cell line NCI H322. The results of these experiments showed that inflammatory mediators affected P-450 expression in both these systems.

In the mouse liver the effects of endotoxin on P-450-dependent enzyme activities were complex, both increases and decreases being observed. In general, constitutive P-450-dependent activities were decreased following endotoxin treatment, whereas the induction of benzphetamine N-demethylase and EROD activity by PB and 3-MC, respectively, was potentiated by low doses of endotoxin. Western blot analysis was used to establish whether altered P-450 isozyme expression accounted for the observed changes in enzyme activities. At low doses, endotoxin caused small, but reproducible, changes in the induced expression of isozymes PB_{3a} and MC_{1b} in mouse liver. The changes in PB_{3a} expression agreed closely with alterations in benzphetamine N-demethylase activity, indicating that elevated expression of this isozyme could have been directly responsible for the increased enzyme activity. Elevated MC_{1b} expression, however, did not account for the potentiation of induction of EROD activity by 3-MC: mechanisms other than an increase in P-450 expression were apparently involved in this potentiation. Suppression of P-450 and associated activities by a high dose of endotoxin appeared to affect all the P-450 isozymes studied. However, all were not equally affected, PB-induced isozymes being suppressed less than constitutive and 3-MC induced forms (Stanley et al (1988)).

An attempt was made to identify a specific agent responsible for the inflammation's effects on P-450s by treating mice with recombinant ifn α plus or minus PB or 3-MC. Endotoxin induces interferon synthesis; it is possible that interferon was at least partly responsible for the observed effects of endotoxin on P-450 expression. In the mouse liver, ifn α mimicked some of the effects of endotoxin, including suppression of constitutive P-450-dependent activities and potentiation of 3-MC induction of EROD activity. However, the effects of ifn α were less marked than those of endotoxin and differed from them in some respects; for example, induction of benzphetamine N-demethylation was suppressed, rather than potentiated, by ifn α. Western blot analysis was again used to establish whether the observed changes in P-450-dependent activities resulted from altered expression of P-450 isozymes. This analysis demonstrated that the isozymes studied had different susceptibilities to this agent. Two constitutively expressed isozymes, PB_1 and MC_{1a}, were significantly suppressed in uninduced mice after ifn α treatment, suggesting that decreased de novo synthesis or increased degradation of these isozymes might be responsible for changes in constitutive enzyme activities. The inducible isozymes studied, PB_{3a} and MC_{1b}, were not significantly affected by ifn α, indicating again that a post-translational mechanism might be involved in regulation of induced P-450-dependent enzyme activities during inflammation.

Post-translational mechanisms may explain changes in induced P-450 activities after treatment with endotoxin or ifn α, including the elevation in EROD activity which is likely to be related to isozyme MC_{1b}. Various mechanisms could affect the activity of this P-450 without changing the total level of expression of the protein; one possibility is that changes in the cell membrane alter the interaction between P-450s.and the reductase enzyme, leading to changes in monooxygenase activity. Altered phosphorylation status may also be involved: the activities of many enzymes are regulated by phosphorylation, and interferons alter phosphorylation processes in target cells (Pestka et al (1987)). Some P-450s are kinase substrates; the phosphorylation of one, rabbit isozyme LM_2 (PB_{3a}) has been studied in detail (Pyerin et al (1983, 1984, 1986), Taniguchi et al (1985)). These studies provided equivocal results concerning the effect of phosphorylation on enzymic activity; dephosphorylation of PB_{3a} increased benzphetamine N-demethylase

activity whereas 7-EC deethylase activity was higher following phosphorylation. Further studies on the phosphorylation status of this isozyme in vivo would indicate whether phosphorylation is a possible mechanism for the decreases in enzyme activities observed when mice are treated with PB plus ifn α. Phosphorylation is an unlikely mechanism for the inflammatory regulation of activities catalysed by isozyme MC_{1b}, since this isozyme is not a substrate for either the cAMP-dependent, calcium/phospholipid-dependent or phosvitin-specific protein kinases (Pyerin et al (1987)). However, this does not preclude a role for other kinases in regulation of MC_{1b}-dependent activities; further work is required in order to understand their role in the effects of inflammatory mediators on this isozyme.

The data obtained in this project agree with recent reports that recombinant interferons suppressed a number of constitutive P-450-dependent activities, including metabolism of B(a)P, benzphetamine, zoxazolamine, hexobarbital, 7-EC and acetaminophen. A human recombinant ifn a hybrid, IFN αAD (BgI), containing residues 1 - 61 of IFNαA and residues 62 - 166 of IFNαD effectively suppressed murine hepatic P-450 levels whereas the parent molecules were less effective; this correlated with the effects of these molecules on the antiviral response of mouse L cells (Singh et al (1982), Parkinson et al (1982), Moore et al (1983), Renton et al(1984)). Considerable data indicates that interferons suppress P-450 levels in the mouse liver, but further work in this system is still required. Studies exist in which, although the capacity of the mouse liver to metabolise model substrates in vitro was suppressed by human IFNαAD (BgI) and murine ifn γ, the effect of the agents on drug clearance in vivo was marginal; this may be due to effects on other drug metabolising enzymes or to a compensatory increase in the amount of hepatic endoplasmic reticulum (Franklin and Finkle (1985, 1986)).

In one of the few published reports concerning the effects of interferons on P-450 induction, treatment with poly IC or tilorone inhibited hepatic induction in the mouse; no potentiation of 3-MC-induction occurred, but MC_{1b}-dependent activities were relatively resistant to the suppressive effects of interferon (Renton et al (1979)). In a more recent report the effects of interferon on P-450 induction were found to be time-dependent; suppression of P-450 induction by interferons was short-lived and 3-MC-induced, but not PB-induced, activities returned to their

induced level a few days after interferon treatment (Crowe et al (1986)). Such a pattern is consistent with the observations made during the present project; 3-MC-induced activities might have recovered from the initial effects of interferon in the three days of interferon treatment which preceeded sacrifice. It appears that P-450 regulation by interferons, at least in the hamster, is even more complex than hitherto suspected. Using a "consensus interferon", IFN α CON1, it was shown that 3 hours after interferon administration to hamsters, P-450, aminopyrine N-demethylase and B(a)P hydroxylase were elevated relative to controls, but that they declined to a significantly suppressed level after 24 hours (Moochhala et al (1989)). A gradual recovery then began, lasting for several days. Similar results were obtained in the lung, spleen and adrenal but not in the kidney where suppression started 3 hours after interferon treatment. Induced P-450s were also studied, but only after 24 hours treatment with 10^6 units of interferon. This dose suppressed induction of all P-450-dependent activities examined, though 3-MC-inducible activities were again the most resistant to suppression by IFN α CON1.

The results discussed above serve to emphasise the complexity of P-450 regulation during inflammation. A great deal of work is still required to clarify this aspect of P-450 regulation: a major problem has been the fact that each investigator used a different model, with widely varying doses of inflammatory agents and time courses. More detailed experiments using a single system, with homologous inflammatory mediators and careful attention to dose and time course will help to clarify the situation. Another way to simplify the study of P-450 regulation by inflammatory agents is to use a cell culture model; this was the second approach chosen for the present project. The human lung tumour-derived cell line NCI H322 was chosen because of the possible importance of interactions between inflammation and P-450 induction in the smoker's lung. Human recombinant cytokines was used in order to circumvent possible problems due to the species specificity of cytokines.

The first two agents tested, endotoxin and TNF, had little effect on EROD activity in NCI H322 cells. This was not surprising in the case of endotoxin, since this agent exerts its effects via the release of cytokines, and does not directly affect P-450 levels in primary rat hepatocytes (Ghezzi et al (1986a)). The lack of a direct effect of TNF was unexpected; TNF is thought to mediate many actions of endotoxin and it suppressed P-450 expression in rat liver and in the livers, lungs, kidneys and

adrenals of CD1 and C3H/HeJ mice (Ghezzi et al (1986b), Duan et al (1988)). The TNF-receptor status of NCI H322 cells is not known; it is possible that they are less responsive to TNF than mouse lung cells. Another possible solution to this anomaly is that TNF might induce the expression of other cytokines, such as IL-1 or ifn γ, which are responsible for the suppression of P-450 levels in the lung.

Constitutive EROD activity in NCI H322 cells was slightly, but significantly, suppressed by IL-1, in agreement with previous studies carried out using a chick embryo/mouse monokine system (Shedlofsky et al (1985)). In the endotoxin-resistant C3H/HeJ mouse strain, IL-1 exerted endotoxin-like effects on hepatic P-450 and 7-EC O-deethylase and suppressed P-450, benzphetamine N-demethylation and EROD by a non-prostaglandin-mediated mechanism (Shedlofsky et al (1987a,b)). The present work demonstrates that this effect of IL-1 is not limited to the liver but may also be observed in a lung-derived system.

The synthetic glucocorticoid, Dex, suppressed EROD in NCI H322 cells both in the presence and absence of BA; this effect, also observed by Falzon et al (1986a)), contrasts sharply with the effect of Dex on BA induction in the liver. Many reports exist of strong potentiation of BA induction by Dex in primery cultured hepatocytes and rat H35-derived cell lines (Gielen and Nebert (1971a,b), Whitlock et al (1974), Goujon et al (1980), Kremers et al (1981), Edwards et al (1984)), Corcos and Weiss (1988)). One explanation for the discrepancy between the two systems is that synergism between Dex and BA could be a liver-specific phenomenon; another is that, in view of recent studies using foetal hepatocytes in which the highest concentration of Dex used was 1μM and half-maximal potentiation was observed at 5nM (Mathis et al (1986a,b)), synergism between Dex and BA could be dose-dependent, with low concentrations of Dex synergising with BA whilst high concentrations oppose BA induction. Further experiments using much lower concentrations of Dex would show whether or not this is the case.

Experiments using interferons showed that ifns α and β had minor effects on EROD activity in NCI H322 cells, but ifn γ dramatically affected both constitutive and induced EROD activity in this cell line, causing at least 50% and 70% suppression of constitutive and induced EROD activity, respectively. There are few reports of interferon regulation of P-450 expression in culture; those that exist were carried

out before the advent of recombinant interferons. In a foetal mouse cell culture model, potentiation of BA induction of B(a)P hydroxylase activity by interferon was described; similar results were obtained in rat foetal cell culture (Nebert and Friedman (1973)). The significance of this finding is hard to assess since P-450 regulation in foetal cell cultures is different from that in adult primary culture and in vivo, and the interferon preparation used was not characterised. Induction of B(a)P and aminopyrine metabolism by poly IC and interferon in primary mouse hepatocytes cultured on floating collagen gels was also reported (Renton et al (1978)); again the results are difficult to assess since primary hepatocyte cultures undergo rapid loss of P-450 during the first 24 hours in culture and this decline continues for several days. Poly IC had no effect on P-450 levels in H35 cells, possibly indicating that the hepatocyte preparations used contained other cell types which were responsible for interferon release in response to poly IC.

The present project represents the most comprehensive survey to date of the effects of inflammatory agents on a P-450-dependent activity in cultured cells. The results indicated that, at least in this lung tumour-derived cell line, glucocorticoids, ifn γ and IL-1 may be important modulators of P-450 expression during inflammation. Although these were the only agents which had statistically significant effects on P-450 expression, all of the cytokines tested caused some suppression of EROD activity; no synergism between BA and inflammatory agents was observed. In these experiments only enzyme activity measurements were performed; further analyses are needed to confirm the results. Unfortunately, the Western blot technique used was insufficiently sensitive to assess such small changes in the expression of P-450s in this cell line, since the P-450 level of induced cells was only detectable after long autoradiographic exposure. Northern blot analysis appears to be a more sensitive technique, and could be used to confirm the observations made herein. In order to test the hypothesis that toxicity towards the cells could account for P-450 suppression by cytokines, the toxicity of each cytokine was tested using the MTT assay. The results showed that toxicity did not account for the onserved effects. It should, however, be noted that the use of the MTT assay to test the toxicity of cytokines has been criticised; anomalous results may be obtained due to cytokine-induced changes in oxidative metabolism by cultured cells (Rashid et al (1988)).

The NCI H322 cell line represents a suitable model for more detailed studies on P-450 regulation by inflammatory agents. In these experiments the maximum tolerated dose of each agent was used: dose-response experiments would show whether lower concentrations of any agent synergise with BA , as occurred in the mouse liver with endotoxin. A detailed time courses would show whether transient elevation followed by suppression of P-450s as observed by Moochhala et al (1989) is a universal phenomenon. Another potentially fruitful area of study is that involving synergism between lymphokines. Synergism between types I and II interferons has been reported (Fleischmann (1982), Schiller et al (1986)), as has potentiation of TNF cytotoxicity by ifn γ (Fransen et al (1986), Schiller et al (1987)). If these cytokines also have synergistic effects on P-450 levels, it is possible that although TNF and type I interferons had relatively minor effects on P-450 levels when administered singly, in combination with ifn γ the result might be almost complete suppression of P-450 expression in NCI H322 cells.

Regulation of lung P-450 expression by inflammatory agents may be important in two situations. The first is the smoker's lung, which is chronically exposed to both P-450 inducers such as B(a)P and inflammatory materials such as carbon particles which might cause release of the inflammatory agents studied in this project. The present results indicate that the most likely outcome of this situation is a reduction in the extent of P-450 induction due to the PAHs in cigarette smoke; however, the interactions between P-450 inducers and inflammation are very complex and may lead to potentiation of induction in some circumstances. An understanding of such interactions is essential if the relationship between smoking and carcinogenesis is to be understood. The role of inflammatory agents in P-450 regulation is also increasingly important in cancer therapy since several cytokines have anti-tumour effects and are now being used in the clinic. A striking success of interferon has been the use of ifn α against hairy cell leukaemia; ifn α is also used to treat a number of other tumours (Smyth et al (1987)). Ifns β and γ have not received as much attention as ifn α, but trials of these agents are also taking place. As well as being used in single agent therapy, interferons are often used in combination with cytotoxic drugs. Complex interactions between interferons and cytotoxic drugs occur; 5-fluouracil toxicity in animals was potentiated or ameliorated by interferon, and interferon had positive and negative effects on cyclophosphamide-induced tumour regression in a hamster lymphosarcoma model (Miyoshi et al (1983), Stolfi et al

(1983), Lee et al (1984)). Mouse xenograft experiments suggested that these effects result from direct action of interferon on the tumour, rather than altered host drug metabolism (Balkwill et al (1984)). In the cancer patient interferon may affect drug metabolism in the tumour, liver and lung, altering responsiveness to cytotoxic drugs in ways which cannot be predicted from animal models. Such interactions may be important as a new generation of drugs enters clinical trials. The P-450-activated lung-specific toxin 4-ipomeanol, for example, is currently undergoing preclinical trials as an anti-cancer agent (Christian et al (1989)); if it is accepted for clinical use, modulation of lung P-450 expression will be a major determinant of response to this drug. Use of combinations of cytokines to give synergistic anti-tumour effects may also have profound effects on the patient's drug metabolising capacity; endogenous TNF was found in cancer patients' serum (Balkwill et al (1987)) emphasising the need for a deep understanding of interactions between exogenous and endogenous cytokines and P-450 inducers when predicting a patient's response to P-450-activated drugs.

The work reported in this chapter showed that inflammation may have significant and complex effects on P-450 expression in experimental systems. More studies are needed before a complete understanding of alterations in P-450 expression in the lungs of smokers and cancer patients exposed to drugs and inflammatory agents is acheived. Cell culture models such as the NCI H322 cell line used in this project represent a simple, reproducible model in which to search for such interactions; promising results can then be confirmed in animal and xenograft models.

Chapter 6

Summary and Future Prospects.

6.1. P-450 expression in human tumour-derived cell lines.

The aim of this project was to characterise P-450 expression and inducibility in a number of human tumour-derived cell lines with a view to choosing one as a model in which to examine the effects of inflammatory mediators on P-450-dependent activities. The hepatoblastoma line HepG2, the non-small cell lung carcinoma lines NCI H322 and NCI H358 and the colon adenocarcinoma lines HT29 and LS174T were examined. The liver cell line was chosen because of the importance of this organ in xenobiotic metabolism and the known effects of inflammatory mediators on hepatic P-450 expression; the lung and colon lines were selected because these organs also express P-450s and are subject to inflammatory disorders which may be involved in tumorigenesis. All the cell lines exhibited levels of P-450-dependent activities which could be detected using the sensitive assay method of Grant et al (1988). The activities of the cell lines differed greatly; basal levels of EROD activity (in pmol product/min/mg cellular protein) ranged from 0.08 in HT29 cells to 5.71 in HepG2 cells. The inducibility of P-450-dependent activities also varied: in the case of EROD activity it ranged from 3 fold in HT29 cells to 261 fold in LS174T cells.

The three enzyme activities measured were readily detectable in HepG2 cells, though PROD and BROD activities were lower than EROD activity. Only BA caused significant P-450 induction in this cell line; EROD activity was the major activity induced, confirming immunochemical evidence that a protein related to rat isozyme MC_{1b} was induced in HepG2 cells by BA. This agreed with the results of studies using human liver microsomes (Wrighton et al (1986), Adams et al (1985), L.M. Forrester, personal communication) and with previous studies on HepG2 cells, which were able to metabolise xenobiotics via P-450-mediated and other pathways (Dawson et al (1985), Bhatt (1986), Sassa et al (1987), Duthie and Grant (1988)). The results

obtained agreed with previous evidence that HepG2 cells expressed alkoxyresorufin and UDP-glucuronyl transferase activities and that EROD activity in these cells was induced in response to BA (Grant et al (1988)).

Both the human lung tumour-derived cell lines studied expressed P-450 levels detectable enzymatically and, in induced cells, by Western blot analysis. NCI H322 cells, thought to be derived from Clara cells, were sensitive to P-450 induction by both BA and Aroclor 1254; BA induced EROD activity (7 fold) whilst Aroclor 1254 induced both EROD activity (6 fold) and BROD activity (3 fold). This implied that at least two isozymes were inducible in NCI H322 cells. The Type II pneumocyte cell line NCI H358 was sensitive to induction of EROD by BA (11 fold); the basal and induced activities of this cell line were lower than those of NCI H322 cells, in agreement with previous evidence that both cell lines express BA-inducible AHH activity (Falzon et al (1986a)). The cell lines were able to activate diethyl-nitrosamine and 4-ipomeanol (McMahon et al (1985), Falzon et al (1986b,c)); they also expressed enzymes involved in conjugation and arachidonic acid metabolism (Weibel et al (1986), Lau et al (1987)). These findings suggest that NCI H322 cells represent a good model for the study of human pulmonary drug metabolism.

The colon adenocarcinoma cell lines studied differed markedly in P-450 expression and inducibility. One, HT29, had barely detectable levels of the activities measured even after induction but in LS174T EROD was more inducible than in any other cell line studied. This difference in inducibility was reflected in Western blot analysis; induction was barely detectable in HT29 cells whereas the induced isozyme was easily detected in LS174T cells. No reports exist of P-450 induction in human colon tumour-derived cell lines; the detection of low levels in HT29 cells was not surprising in view of their undifferentiated phenotype but the strong inducibility of P-450s in LS174T cells was unexpected. This cell line will be an interesting model for the study of colonic P-450 regulation, but it is important to question how its inducibility arose; it may represent a subpopulation of cells which are present in normal colon at very low levels, in which case it is a good model for P-450 regulation in this cell type. Alternatively, the great inducibility of P-450s may have arisen during tumour development or adaptation to culture, in which case the results of experiments on P-450 induction, though interesting, may not be relevant to the situation in normal human colon. The cell lines HT29 and LS174T are potentially useful models in which to examine the role of differentiation in human

colonic P-450 regulation. LS174T cultures consist of a mixture of mucin-secreting and non-mucin-secreting cells which are separable (Kuan et al (1987)). Colon tumours which produce large amounts of mucin have a poor prognosis; studies on P-450 expression in the two types of LS174T cells might clarify the role of drug metabolism in this phenomenon. LS174T cells may also be induced to undergo differentiation by treatment with sodium butyrate (Gum et al (1987)) and could be used to examine the role of differentiation in colon P-450 regulation. Similarly, certain subclones of HT29 may be induced to differentiate (Laboisse et al (1988), Phillips et al (1988)). It would be interesting to discover whether P-450 inducibility in this cell line was restored as a result of growth under conditions which favour differentiation.

In order to discover whether the same P-450 isozymes were expressed in tumour-derived cell lines, tumour samples and normal tissue, a group of human lung and colon tumours was studied. Histologically normal tissue from each patient was also examined. Problems were experienced with the analysis since the levels of P-450 expression in these samples were very low, but proteins cross-reacting with antisera raised against isozymes PB_1, PB_{2c}, PB_{3a} and MC_{1b} were detected, and all three enzyme activities were measurable. The immunochemical detection of four P-450s in human lung and colon samples contrasted with the cell line results, in which only isozyme MC_{1b} was detectable even after induction. The isozyme most consistently detected in the human lung and colon samples was PB_1, which was present in all the samples studied. Expression of proteins related to PB_{2c} varied greatly between samples; one lung tumour contained an exceptionally high level of two such proteins. It was intriguing that neither the lung cell lines nor the human lung samples expressed high levels of isozyme PB_{3a}; instead, human lung samples appeared to express the related isozyme PB_1. Low levels of a protein related to rat MC_{1b} were detected in the human lung and colon samples; as in the tumour-derived cell lines, the protein detected had slightly faster mobility than purified rat MC_{1b}. There was no evidence for induction of this P-450 in lung or colon samples, which was surprising since cigarette smoking was expected to induce isozyme MC_{1b} in the lung cancer patients. The profile of P-450-dependent activities expressed by the human samples differed from that of the cell lines: in human samples the most

readily detected activity was BROD whereas in the cell lines it was EROD. This may have been due to differential loss of activities during freezing and thawing; a more interesting possibility is that the cell lines had regressed to a less differentiated phenotype. There is evidence that undifferentiated foetal hepatocytes retain more P-450 expression than do adult hepatocytes; higher levels of 3-MC-inducible P-450 expression were observed in foetal cells, and these were further inducible by both PB- and 3-MC-type inducing agents (Gielen and Nebert (1971a,b, 1972), Goujon et al (1980)). It is possible that dedifferentiation of cultured human lung tumour cells led to expression of a phenotype similar to that of undifferentiated foetal cells.

No consistent differences in P-450 expression between normal and tumour samples were detected by Western blot analysis. In some cases the levels of P-450-related proteins were elevated in the tumour but in others there was little difference in P-450 expression between normal and tumour material. The number of samples studied was not sufficiently large to draw conclusions about the relationship between tumour type and P-450 expression. The lung sample which labelled most intensely was from a male patient bearing a poorly differentiated squamous cell carcinoma; however, the group included tumours of this type which had lower levels of P-450 expression. The colon sample with the highest levels of P-450-related proteins came from a female. Only one other patient in the colon cancer group was female; further studies would be required to show whether there is a sex difference in human colonic P-450 expression. Some differences in P-450-dependent enzyme activities between normal and tumour samples were detected: the suppression of EROD activity in lung tumours relative to normal lung parenchyma agreed with evidence that AHH activity was significantly lower in lung tumours than in normal lung parenchyma (De Flora et al (1987)). Further studies are required in order to assess the significance of the finding that BROD activity was significantly reduced in colon tumours relative to normal mucosa from the same patients.

In order to establish the NCI H322 cell line as a suitable model for further studies on P-450 regulation, further characterisation of the induction process was carried out. Induction was shown to be reproducible and statistically significant and could be observed as an increase in the steady-state level of mRNA as well as MC_{1b} protein. The optimum concentration of BA for induction was 3 - 5μg/ml (13 - 22μM). The

growth medium giving the greatest ratio of induced to basal activity was RPMI; this medium was selected for further experiments on P-450 induction in NCI H322 cells. Of the three media tested, the medium supporting the highest basal EROD activity was William's E medium, which was previously shown to support the maintenance of P-450 expression in primary hepatocytes and HepG2 cells (Grant et al (1985), Steward et al (1985), Doostdar et al (1988)).

For complete characterisation of P-450 induction in NCI H322 cells, the isozymes induced by BA and Aroclor 1254 must be identified unequivocally. The enzyme detected by Northern blot, Western blot and enzymatic analysis in NCI H322 cells could be either MC_{1a} or MC_{1b}. In order to identify it precisely, two approaches are available. The first is to perform antibody inhibition experiments using a range of monoclonal antibodies raised against these two isozymes in order to discover which antibodies inhibit EROD activity in NCI H322 cells. The second is to synthesise oligonucleotide probes complementary to the two genes in regions where their sequence differs and use these to probe Northern blots of control and BA-induced NCI H322 cellular RNA. Further analysis using immunohistochemistry would indicate whether all members of a NCI H322 population are equally inducible. If induction proved to be heterogeneous, it might be possible to select a more inducible sub-line by fluorescence activated cell sorting following treatment with an MC_{1b} substrate such as B(a)P or EROD. Few human cell lines have been shown to contain a functional Ah receptor; it would be of interest to compare the properties of the Ah receptor in NCI H322 and any "superinducible" sub-line. Unlike rabbit lung parenchymal cells, which express high levels of isozyme PB_{3a} (Wolf et al (1980)), and human lung cells which appear to express isozyme PB_1, NCI H322 cells did not express significant levels of PB-inducible isozymes. The reason may be a defect in P-450 regulation in this cell line; alternatively, the low level of these isozymes may be due to the absence from culture medium of trans-acting factors responsible for the regulation of pulmonary P-450 expression. Examination of the effects of different mediators, alone and in combination, on the spectrum of P-450s expressed by NCI H322 cells might facilitate the identification of such factors.

This section of the project indicated that the human lung tumour-derived cell line NCI H322 is appropriate for the study of human pulmonary P-450 regulation at the cellular level. The expression and inducibility of PAH-inducible P-450s in this cell

line renders it a potentially useful model for studying pulmonary carcinogen metabolism. Comparison of this cell line with NCI H358 cells may clarify the roles of the Clara cell and the Type II pneumocyte in pulmonary carcinogen activation and detoxication. However, the proviso that both cell lines are of tumour origin and have adapted to growth in culture should be noted; their metabolic properties may differ from those of normal lung cells. For this project, the NCI H322 cell line was selected for examinating the effects of P-450 induction on activation of cytotoxins and of inflammatory mediators on constitutive and inducible P-450 expression.

6.2. Effects of P-450-inducing agents on susceptibility to cytotoxins: Use of the MTT assay.

To characterise further the effects of P-450-inducing agents on NCI H322 and HepG2 cells, the MTT assay was used to assess the cytotoxicity of the compounds used in induction experiments. The behaviour of the cell lines was first characterised in detail and the assay modified slightly to allow measurement of the cytotoxicity of hydrophobic compounds. The chosen cell lines proved to be appropriate for use in the MTT assay since they grew consistently on 96-well plates and the relationship between the OD obtained after MTT treatment and the number of cells present approximated to linearity. By plating out suitable numbers the cells were kept in logarithmic growth throughout the assay. Most of the P-450-inducing agents tested caused little toxicity to either of the cell lines, though PB did cause 50% cell death at about 4mM. This was surprising since 3mM PB was reported to have a beneficial effect on the survival of hepatocytes in primary culture (Miyazaki et al (1985)). Aroclor 1254 actually enhanced the growth of HepG2 cells relative to cells treated with DMSO: the mechanism of this effect is unknown and merits further study.

The suggestion arose that the MTT assay could be used to assess changes due to P-450 induction in the sensitivity of cells to cytotoxins. Two compounds, B(a)P and cyclophosphamide, were used to test this hypothesis: B(a)P is activated by MC1b whereas cyclophosphamide is thought to be metabolised by PB3a. The MTT assay was used to compare the cytotoxicity of the compounds towards BA- and vehicle-treated HepG2 and NCI H322 cells. It was predicted that BA treatment would increase the sensitivity of the cell lines to the cytotoxicity of B(a)P but not cyclophosphamide. In recent experiments, 3-MC-treatment of HepG2 cells increased their sensitivity to

the toxic effects of aflatoxin B_1 (Herweijer et al (1988)). In the present study, however, treatment of HepG2 cells with BA appeared to reduce their susceptibility to B(a)P cytotoxicity. Protection of the cell from B(a)P toxicity may have occurred as a result of increased detoxification or decreased activation, either of which might be P-450-mediated. Efficient DNA repair or free-radical scavenging systems could also be involved in the protection mechanism.

One possibility was that BA treatment induced a P-450 which metabolised B(a)P to a non-toxic product leading to efficient detoxification. Such a mechanism was proposed for protection against bromobenzene-induced liver necrosis by 3-MC treatment (Jollow and Smith (1977)). This mechanism would not be consistent with the presumed role of the 3-MC-inducible P-450s in B(a)P metabolism, but it should be noted that the properties of human isozyme MC1b are poorly characterised and could differ from those of the equivalent isozyme in other species. Another possibility was that cells expressing high levels of MC_{1b} were killed by BA treatment, so that the population which finally encountered B(a)P contained little AHH activity. If this were the case, the cells which survived BA-treatment would be expected to contain low levels of EROD activity at the time of exposure to B(a)P. However, the cells which survived the plating out procedure continued to be induced by BA, expressing high levels of EROD activity. Other explanations for the resistance of these cells to B(a)P must therefore be considered. The third possibility was that BA induced detoxifying enzymes in the HepG2 cells or selected a population of cells which already had high levels of detoxifying activities. The primary metabolites of B(a)P undergo further metabolism by various enzymes, many of which are PAH-inducible; these include conjugating enzymes such as GSTs and UDPGTs (Burchell (1981), Mannervik (1985)). Transfection of the GST Ya gene was recently shown to protect cells from the cytotoxic effects of B(a)P diol epoxides (Pitot and Sato (1988)); several GSTs and UDPGTs have been shown to be inducible by 3-MC ((Bock et al (1973), Hales and Neims (1977), Wishart (1978), Pickett et al (1984), Dolan et al (1988)). It is not clear whether protection of HepG2 cells from B(a)P toxicity by induction of detoxifying enzymes is possible. It is thought that HepG2 cells contain efficient detoxifying systems (Diamond et al (1984), DiGiovanni et al (1984)), but the role of UDPGTs in conjugation in HepG2 cells is unclear and little is known about the regulation of GSTs in this cell line (Diamond et al (1980), Dawson et al (1985), Grant et al (1988), A.D.Lewis, PhD Thesis

(1988)). Measurement of GST induction in HepG2 cells would indicate whether induction of these enzymes could be involved in protection against B(a)P toxicity as a result of pretreatment with BA.

The lung cell line NCI H322 was resistant to the cytotoxicity of B(a)P, regardless of whether the cells had been pretreated with BA, in contrast with published results (Keifer et al (1988)). The major route of detoxification of B(a)P metabolites in human lung may involve sulphatases, GSTs and UDPGTs (Harris et al (1977), Mehta and Cohen (1979), Gibby et al (1981)). The role of the high level of GST activity found in NCI H322 cells is not clear (Wiebel et al (1986), Keifer et al (1988)); removal of B(a)P-DNA adducts may also be important in human lung tumour-derived cell lines (Cerutti et al (1978), Feldman et al (1978)).

Cyclophosphamide was toxic to both HepG2 and NCI H322 cells suggesting that the cells did express isozyme PB3a, in spite of their low level of PROD activity and apparent absence of PB3a protein. BA-treatment had no effect on their susceptibility to cyclophosphamide, implying that MC_{1b} was not involved in cyclophosphamide activation, or that both activating and detoxifying systems were equally induced by BA. The fact that the population of HepG2 cells surviving BA treatment did not have altered susceptibility to cyclophosphamide showed that the protection against B(a)P toxicity caused by BA treatment was not a universal phenomenon affecting susceptibility to all cytotoxic agents.

Part of the reason for performing this study was the hope that induction of P-450-dependent activation of anticancer drugs would increase the sensitivity of refractory tumours to chemotherapeutic agents. The idea that induction of P-450s in the liver might improve the therapeutic response to drugs such as cyclophosphamide arose in the 1960s but proved to be erroneous when it was shown that modulation of cyclophosphamide metabolism did not affect its therapeutic efficiency, probably due to altered clearance of alkylating metabolites (Sladek (1971, 1972 a,b, 1973), Field et al (1973), Struck et al (1984)). The rationale behind the present work was that if P-450-dependent metabolic activation was made to take place in the tumour itself, in the hope that the short-lived reactive intermediates generated would attack tumour cells without entering the bloodstream. However, the results were not as predicted: pretreatment with BA caused a decrease rather than an

increase in the susceptibility of cultured cells to B(a)P cytotoxicity, illustrating the importance of achieving a complete understanding of the metabolism of the compound of interest before attempting to predict the outcome of a change in P-450 expression. The idea of inducing P-450- dependent drug activation in order to increase the sensitivity of tumours to chemotherapy does merit consideration; human tumour-derived cell lines which are responsive to induction of P-450s would be valuable in the early stages of such a project, although it should be noted that routes of drug metabolism may differ between the tumour and the cell line and between different tumours. In order for this approach to chemotherapy to be successful, a thorough understanding of the metabolic pathways followed by the drug in question is essential; the actions of the inducing agent should also be well characterised and it should only induce enzymes involved in the activation of the candidate drug. A means must be devised to deliver the inducing agent directly to the tumour and to prevent drug activation in the liver. The last but probably most difficult requirement is that the inducing agent must fulfil the legal criteria for use as a drug. These targets will be difficult to achieve, but are worth aiming for if the outcome is an improvement in the reponse of refractory tumours to chemotherapy.

6.3. Effects of inflammatory mediators on P-450 expression.

The response of NCI H322 cells to P-450 inducers having been characterised, it was possible to study the effects of inflammatory mediators in this cell line. The cell culture system was used to supplement work carried out using CBA mouse liver. First, experiments were carried out to confirm and extend previous work which suggested that low doses of endotoxin or ifn α, administered concomitantly with PB or 3-MC, could actually potentiate the induction process. In the mouse liver the effects of endotoxin on P-450-dependent enzyme activities were complex. In general, constitutive P-450-dependent activities were decreased following endotoxin treatment, whereas induction of benzphetamine N-demethylase and EROD activity by PB and 3-MC, respectively, was potentiated by low doses of endotoxin. Western blot analysis was used to establish whether alterations in P-450 isozyme expression accounted for the observed changes in enzyme activities. Low doses of endotoxin caused a slight elevation in the induced expression of isozymes PB_{3a} and MC_{1b} in

mouse liver; the suppression of P-450 and associated activities by a high dose affected all the P-450 isozymes studied (Stanley et al (1988)). In an attempt to identify a specific inflammatory agent which affected P-450s, mice were treated with recombinant ifn α with and without PB or 3-MC. Ifn α mimicked some of the effects of endotoxin , including suppression of constitutive hepatic P-450-dependent activitities and potentiation of 3-MC induction of EROD. However, the effects of ifn α were less marked than those of endotoxin and differed from them in some respects: for example, induction of benzphetamine N-demethylation by PB was suppressed, rather than potentiated, by ifn α. Western blot analysis demonstrated that suppression of P-450 isozyme expression occured as a result of treatment with ifn α, but that the isozymes studied had different susceptibilities to this agent. Two constitutively expressed isozymes, PB_1 and MC_{1a}, were suppressed in uninduced mice after ifn α treatment, but the two inducible isozymes studied, PB_{3a} and MC_{1b}, were not significantly affected by ifn α in uninduced or induced mouse liver.

Post-translational mechanisms may explain the changes in induced P-450 activity after treatment with endotoxin or ifn α. An altered interaction between P-450 and reductase, causing changes in monooxygenase activity, is one possibility. Changes in phosphorylation status may also be involved; interferons are known to alter the phosphorylation status of target cells (Pestka et al (1987)). P-450s are kinase substrates, but isozyme MC_{1b} was not a substrate for several well-understood protein kinases tested (Pyerin et al (1983, 1984, 1986, 1987), Taniguchi et al (1985)); this does not preclude a role for kinases in regulation of MC_{1b}-dependent activities, but further work is needed in order to understand the possible role of phosphorylation in P-450 modulation by inflammatory mediators.

The data obtained in this study were in general agreement with a number of reports concerning the effects of recombinant interferons on constitutive P-450-dependent activities (Singh et al (1982), Parkinson et al (1982), Moore et al (1983), Renton et al (1984)). Few reports exist concerning the effects of interferons on P-450 induction: potentiation of hepatic P-450 induction in the mouse has not been reported although the 3-MC-inducible P-450 family does appear to be relatively resistant to interferon-mediated suppression (Renton et al (1979), Crowe et al (1986), Moochhala et al (1989)). A great deal of work is still required in order to

understand this aspect of P-450 regulation: more detailed experiments using a single system, with homologous inflammatory mediators and careful attention to dose and time course should help to clarify the situation.

The results obtained using the mouse liver as a model were difficult to interpret. In order to simplify the situation, further studies were carried out using the cell line NCI H322, whose pattern of P-450 expression was characterised for this reason. The complexity of the experimental system was minimised by adding highly purified, human recombinant DNA-derived cytokines directly to the medium. Endotoxin and TNF did not affect EROD activity in NCI H322 cells; this was not surprising in the case of endotoxin, but the lack of a direct effect of TNF was unexpected. The TNF-receptor status of NCI H322 cells is not known; they may be unresponsive to TNF. Alternatively, TNF may induce the expression of other cytokines which mediate P-450 suppression in the lung. Constitutive EROD activity in NCI H322 cells was slightly suppressed by IL-1, demonstrating that P-450 suppression by IL-1 is not limited to the liver. Dex suppressed EROD activity in NCI H322 cells both in the presence and absence of BA. This effect was previously observed in NCI H322 cells (Falzon et al (1986)), but is in sharp contrast with the effect of Dex on BA induction in the liver (Gielen and Nebert (1971a,b), Whitlock et al (1974), Goujon et al (1980), Kremers et al (1981), Edwards et al (1984), Mathis et al (1986 a,b), Corcos and Weiss (1988)). Synergism between Dex and BA may be liver-specific or may alternatively be dose-dependent, low concentrations of Dex synergising with BA whilst high concentrations oppose BA induction. Type I (α and β) interferons had minor effects on EROD activity in NCI H322 cells, but ifn γ dramatically suppressed both constitutive and induced EROD activity. The few previous reports of regulation of P-450s in culture by interferons preceeded the advent of recombinant interferons; in foetal cell culture models, both direct P-450 induction and potentiation of BA induction by interferons were reported (Nebert and Friedman (1973), Renton et al (1978)), but the significance of these findings is difficult to assess since P-450 regulation in foetal cell cultures is different from that in either adult primary culture or in vivo, and crude interferon preparations were used.

The present project represents the most comprehensive survey to date of the effects of inflammatory agents on a P-450-dependent activity in cultured cells. The results indicate that, at least in this cell line, ifn γ, IL-1 and glucocorticoid hormones are

important modulators of P-450 expression during inflammation. All the cytokines tested caused some suppression of EROD activity; no potentiation of induction was observed, and the toxicities of the cytokines could not account for these effects. Unfortunately, the Western blotting technique used was insufficiently sensitive to assess such small changes in the expression of P-450s in this cell line, but Northern blot analysis would help to confirm these observations at the steady-state mRNA level. The NCI H322 system represents a suitable model for more detailed studies on P-450 regulation by inflammatory agents. In these experiments the maximum tolerated dose of each agent was used: dose-response experiments would show whether synergism with BA occurs at lower concentrations. Detailed time courses would show whether transient elevation followed by suppression of P-450s occurs in lung cells. Another potentially fruitful area of study is that involving synergism between lymphokines (Fleischmann (1982), Fransen et al (1986), Schiller et al (1986, 1987)). Although TNF and type I interferons had relatively minor effects on P-450 levels when administered as single agents, a dramatic reduction in P-450 expression in NCI H322 cells might occur if they were administered in combination with ifn γ.

Various factors may explain the discrepancy between the results obtained in mouse liver and in cultured human lung tumour cells. The cytokine concentrations used in vitro may have been too high; in mouse liver only low doses of endotoxin caused potentiation. Alternatively, the effects observed in mouse liver may have been indirect, involving intracellular messengers travelling between different cell types, or tissue- or species-specific. More information on P-450 regulation during inflammation, both in the liver and lung, is needed before this aspect of P-450 biology is understood. The use of cell lines represents one approach which should be pursued, but any findings made in vitro must be extended with studies in animals.

Two circumstances exist in which regulation of pulmonary P-450 expression by inflammatory agents may be important. The first is the smoker's lung, which is chronically exposed to both P-450 inducers such as B(a)P and inflammatory materials such as carbon particles. The present results indicate that the most likely outcome of this situation is a reduction in the extent of P-450 induction due to the PAHs in cigarette smoke; however, interactions between P-450 inducers and inflammation can lead to potentiation of induction in some circumstances. An

understanding of these interactions is essential if the relationship between smoking and carcinogenesis is to be fully understood. A second area in which the role of inflammatory agents in P-450 regulation is of increasing importance is cancer therapy. A number of cytokines have been shown to have anti-tumour effects and these are now being used in the clinic, often in combination with cytotoxic drugs. In the human cancer patient, interferon may have complex effects on drug metabolism in the tumour, liver and lung. Such interactions may prove to be important as a new generation of activatable drugs enters clinical trials: for example, if 4-ipomeanol is accepted for chemotherapy (Christian et al (1989)), modulation of pulmonary P-450 expression during lung inflammation will be a major determinant of clinical response. The work reported in this chapter showed that inflammation may have significant and complex effects on P-450 expression in experimental systems. Many more studies are needed before a complete understanding of the interactions between drugs and inflammatory agents can be acheived. Cell lines such as NCI H322 represent simple, reproducible systems in which to search for such interactions: promising results can then be confirmed in animal and xenograft models.

6.4. Future prospects.

In this thesis the regulation of P-450s in human tumour-derived cell lines was characterised in number of ways. Initial screening experiments involving Western blot and enzymic analysis made it possible to choose a cell line, NCI H322, which was suitable for the study of pulmonary P-450 regulation. Induction of isozyme MC1b was shown to involve an increase in steady-state mRNA expression, and further experiments established suitable conditions for induction (5μg/ml BA in RPMI medium supplemented with 10% FCS). In order to study P-450-dependent metabolic activation of drugs and carcinogens, the behaviour of HepG2 and NCI H322 cells in the MTT assay was characterised. The results of cytotoxicity assays using B(a)P were unexpected in that, rather than increasing the cells' susceptibility to B(a)P toxicity, pretreatment with BA was protective for HepG2 cells and did not affect the response of NCI H322 cells. A mechanism involving the induction of detoxifying enzymes was proposed for this effect. Assays using cyclophosphamide confirmed that both cell lines were capable of activating this drug and that induction of MC1b did not affect susceptibility to cyclophosphamide. Following extensive characterisation of the response of NCI H322 cells to P-450-inducing agents, this cell line was deemed to be a suitable model in which to study P-450 regulation by

recombinant lymphokines. The response of the cell line to inflammatory mediators was compared to that of the CBA mouse liver. In the mouse, constitutive P-450 expression at the enzymic and protein level was suppressed by a high dose of endotoxin (25μg/day) and by ifn α, but P-450 induction by 3-MC and PB appeared to be potentiated by low doses of endotoxin. No evidence for potentiation of P-450 induction was observed in NCI H322 cells; the most dramatic effect observed in the cell line was that of ifn γ which suppressed EROD activity to 47.5% and 27.7% of the non-ifn γ treated sample in controls and BA-treated cells, respectively. This suppression was not due to toxicity, since 86% cell survival occurred in MTT assays in the presence of the concentration of ifn γ used (1000u/ml).

In order to confirm the cell line NCI H322 as a model for human pulmonary P-450 regulation by ifn γ it is necessary to demonstrate that it expresses the same response mechanisms as human lung. It is known that ifn γ binds to a cellular receptor distinct from that for ifns α and β, although the mechanism of signal transduction by the ifn γ receptor is poorly understood; there is some evidence that direct delivery of ifn γ to the inside of the cell can also elicit a response (Langer and Pestka (1988)). A first step towards confirming the relevance of NCI H322 cells for further studies would be to compare the level of ifn γ receptors on NCI H322 cells and normal human lung cells, especially Clara cells, by examining the binding of ifn γ to the cell membrane and by identifying the receptor itself. In one study, ifn γ receptors were found on the majority (71/76) of human tumour cell lines examined: the affinities of receptors on different cell types were similar (~$2.84 \pm 0.85 \times 10^{-11}$M) but the number of receptors per cell varied widely (from 1000 - 20,000/cell) (Ucer et al (1986)). Unfortunately no lung tumour- derived cell lines were included in the study: it is likely that NCI H322 cells, like the other cell lines studied, have a receptor with a dissociation constant of about 3×10^{-11}M, but it is impossible to predict the number of receptors on each cell. The ifn γ receptor has been purified; it is a membrane protein with a molecular weight of approximately 95,000 to which antibodies have been raised in mice (Rubenstein et al (1987)). In order to confirm the involvement of ifn γ receptor binding in P-450 suppression, it should be demonstrated that antibodies to both the receptor and ifn γ itself are able to inhibit this response.

Two aspects of P-450 regulation by ifn γ merit further study in NCI H322 cells, namely the dose-response and time course of suppression. In the present study a high concentration of ifn γ (1000u/ml) was used. However, the ifn γ receptor reaches 50% saturation at 300u/ml whilst half-maximal biological activity is observed at only 1u/ml (Rubenstein et al (1987)). A dose-response curve using lower concentrations of ifn γ would help to prove the hypothesis that ifn g-induced P-450 suppression in NCI H322 cells is a physiological phenomenon rather than an artifact caused by the application of excess ifn γ. This experiment might also reveal the kind of biphasic response observed with endotoxin in the mouse liver, with potentiation of P-450 induction at low concentrations giving way to suppression at high concentrations. It appears that the duration of exposure of cells to ifn γ is critical in determining which responses occur: some responses, including hydrogen peroxide production, Ia antigen induction and immunoglobulin Fc receptor expression occur within minutes of exposure of macrophages to ifn γ, whereas development of tumoricidal activity requires continuous exposure for at least 4 hours, implying that several rounds of receptor occupancy are needed (Celada (1988)). A detailed time course would help to determine into which class of responses P-450 suppression falls, thus indicating further directions for study of the phenomenon of ifn γ-mediated P-450 suppression in NCI H322 cells.

In order to test the hypothesis that ifn γ is a true mediator of the effects of inflammation on pulmonary P-450 in vivo it is, of course, essential to demonstrate that similar effects are observed in the lungs of experimental animals. In line with previous studies on hepatic P-450 regulation one should examine the effects on pulmonary P-450 expression of known inducers of ifn γ, making the prediction that the magnitude of P-450 suppression will correlate with serum ifn γ levels attained. The mouse is the best species to use for these experiments since recombinant murine interferons are readily available. Recombinant DNA-derived murine ifn γ should be administered to mice and its effects on pulmonary P-450 measured. In these experiments great care must be taken to ensure that the duration of exposure to ifn γ is optimised, since this agent has a short half-life in vivo.

The reason for depression of the P-450 system by interferon is still unclear: cell culture models such as the NCI H322 system characterised in this thesis, together

with the liver cell line HepG2, have the potential to resolve this problem by clarifying the intracellular signalling pathways involved. P-450 modulation by interferon is thought to involve either suppression of de novo P-450 synthesis or increased degradation of P-450 haem. Two enzymes, haem oxygenase and xanthine oxidase have been implicated in mechanisms involving haem degradation.

The role of haem oxygenase in P-450 suppression has been a subject of debate for some years. This enzyme is involved in the degradation of excess haem within cells and is induced by several compounds which suppress P-450 expression (Kikuchi and Yoshida (1983)). A reciprocal relationship between the levels of P-450 and haem oxygenase was observed during P-450 suppression by interferon inducers and during the first 24 hours of hepatocyte culture, leading to the suggestion that haem oxygenase caused P-450 suppression by degrading the haem moiety of P-450 (Bissell et al (1974), Renton et al (1979), Mannering et al (1980)). However, this proposal failed to take into consideration two facts: P-450 degradation preceded haem oxygenase induction by several hours and high P-450 levels could be maintained in cultured hepatocytes in the presence of haem oxygenase (Bissell and Hammaker (1976), Paine and Legg (1978)). These results suggested that if a causal relationship between these two phenomena did exist, the release of P-450 haem was likely to be responsible for induction of haem oxygenase rather than vice versa.

Involvement of xanthine oxidase in P-450 suppression was proposed on the basis of evidence that this enzyme was interferon-inducible and generated free radicals such as the superoxide anion which could suppress P-450 activity either directly or via lipid peroxidation (Ghezzi et al (1984), Ali et al (1985), Koizumi et al (1986)). It was claimed that the extent of P-450 suppression by recombinant interferons correlated with their ability to induce xanthine oxidase, and that treatment with the free radical scavenger N-acetyl cysteine or inhibition of xanthine oxidase by allopurinol prevented P-450 suppression in response to poly IC (Ghezzi et al (1985,1986c)). However, detailed evaluation did not support these findings: the results of the N-acetyl cysteine experiment were not reproducible, and studies in which either P-450 or xanthine oxidase activities were modulated indicated a casual, rather than causal, relationship between xanthine oxidase induction and P-450 suppression (Deloria et al (1985), Mannering et al (1988)). Use of cultured cells should facilitate examination of the potential role of xanthine oxidase in P-450 suppression due to interferon. A detailed time course of the effects of

interferon on P-450 and xanthine oxidase in NCI H322 and HepG2 cells would establish the true temporal relationships between effects on these enzymes and a dose response experiment would demonstrate whether the responses of the enzymes correlate at all doses of interferon. Treatment of the cells with interferon in the presence of xanthine oxidase inhibitors (eg. allopurinol, tungstate), free radical scavengers (eg. N-acetyl cysteine) and protectors of P-450 haem (eg. SKF 525A) would also help to extend our understanding of the role of this enzyme.

Alterations in the rate of de novo P-450 protein synthesis have also been implicated in the effects of interferon. Metabolic labelling studies indicated that the steady state level and rate of synthesis of P-450 proteins were reduced after treatment with interferon inducers (Zerkle et al (1980), Singh and Renton (1984), Gooderham and Mannering (1986)). In this project Western blot analysis was used to demonstrate that the steady-state levels of several P-450 proteins were suppressed following treatment of mice with endotoxin or ifn α, but P-450 levels in NCI H322 cells were too low for this result to be confirmed in the cell line. It appears that alterations in protein synthesis are indeed involved in P-450 modulation by interferon; the next step is to discover whether this effect is mediated at the level of RNA expression. Northern blot analysis of total cellular RNA from control and interferon-treated NCI H322 and HepG2 cells would reveal any alteration in the steady-state concentration of P-450 mRNAs, and studies using techniques such as the nuclear run-off assay would show whether interferon alters the rate of P-450 mRNA synthesis. In order to confirm all these results, the effects of interferon on P-450 expression should be compared with effects on a known interferon response such as major histocompatibility complex Class II induction.

Since the signal transduction pathways of interferon are as yet poorly understood, it is difficult to suggest experiments which would confirm that P-450 suppression proceeds via the normal intracellular pathways activated by interferons. However, a number of approaches are possible. It appears that internalisation of interferon and its receptor is required for some interferon responses; comparison of the effects of interferon on P-450 suppression in NCI H322 and HepG2 cells at 4^{o}C (a temperature at which internalisation is so slow as to be negligible) and 37^{o}C would demonstrate whether this is the case for P-450 suppression. Various second messenger systems, including the cyclic AMP-dependent protein kinase and protein

kinase C systems and altered calcium flux, have been implicated in the signal transduction pathway of interferons (Langer and Pestka (1988), Rubenstein et al (1987)). It would therefore be of interest to discover whether dibutyryl cAMP, phorbol myristate acetate or A23187 (a non-hydrolysable analogue of cyclic AMP, a protein kinase C inducer and a calcium ionophore, respectively) could mimic the effects of interferon on P-450 expression in cell lines. Finally, sequence analysis of the 5' regions of cloned P-450 genes would confirm whether these genes contain the necessary regulatory elements for interferon responsiveness.

In this project a specific aspect of the endogenous regulation of P-450 expression, regulation during inflammation, was considered in some detail using the human non-small cell lung carcinoma cell line NCI H322 as a model. This cell line, together with the hepatoma line HepG2 and the colon adenocarcinoma line LS174T, will be valuable for the study of other aspects of the endogenous regulation of P-450 expression. The effects of a variety of molecules which regulate cellular behaviour could usefully be studied using these cell lines, leading to a more complete understanding of the interactions between P-450s and cellular growth, differentiation and metabolism. HepG2 cells have already been used to study the regulation of acute phase protein synthesis by hepatocyte stimulating factors, a group of peptide mediators including ifn β_2 which collaborate with IL-1 and glucocorticoid hormones in regulating the hepatic acute phase response (Baumann et al (1986,1987), Gauldie et al (1987)); elucidation of the effects of these peptides on P-450 expression would extend the present work on P-450 regulation during inflammation and help to clarify the relationship between the acute phase response and P-450 regulation. Hepatomitogens such as epidermal growth factor, transforming growth factor α, endothelial cell growth factor 1 and hepatopoietins A and B are also of great importance in the regulation of hepatic growth and differentiation (Michalopoulos (1989)); these may significantly affect P-450 expression, which is known to be suppressed in preneoplastic foci in the rat liver (Buchmann et al (1987)). In the lung, a clinically important group of growth regulators includes bombesin and bombesin-like peptides, autocrine growth factors which are released by small cell lung carcinomas (Cuttitta et al (1985), Carney (1987)); the effects of these peptides on the metabolism of drugs by the lung are unknown, but merit further study. Endogenous regulation of P-450 expression in the colon is also important, but poorly understood; LS174T cells would be an

appropriate model in which to extend previous work on the effects of gastrointestinal hormones such as gastrin and secretin (Strobel et al (1980)). This list is by no means comprehensive; there is evidence that the majority of hormones and growth factors studied to date modulate P-450 expression, and it will be many years before the endogenous regulation of these essential xenobiotic metabolising enzymes is fully understood.

It is hoped that the experiments described in this thesis have helped to establish human tumour-derived cell lines as appropriate systems for the study of P-450 regulation. The use of continuously cultured cell lines of both human and animal origin complements in vivo studies by making it possible to examine the direct effects of specific mediators in a closely defined system. Such an approach has the advantage of simplicity compared with studies in intact animals, although the cell culture system cannot be studied in isolation and all results must be confirmed in vivo. Experiments on intact animals are essential to the study of long-range interactions, particularly those involving several tiers of blood-borne intercellular messengers. However, an integrated approach using both continuously cultured cell lines and experimental animals can be both informative and economical, and avoids causing suffering to an excessive number of animals. This is particularly important in the present climate of public opinion which is predominantly opposed to any unnecessary use of animals in the laboratory.

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Cancer Letters 32 (1986) 327 - 334.

Appendix 1.
Sources of Chemicals.

Supplier	Chemicals
Aldrich Fine Chemicals Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL.	B(a)P.
Amersham International PLC, UK Sales Office, Lincoln Place, Green End, Aylesbury, Buckinghamshire, HP20 2TP.	α^{32}P-dCTP, Hybond N membrane, ^{125}I-Protein A.
Anderman & Company Ltd. Laboratory Supplies Division, 145, London Road, Kingston-upon-Thames, Surrey, KT2 6NH.	0.45μM nitrocellulose filters.
BDH, Macfarlane Robson Ltd, Burnfield Ave, Thornliebank, Glasgow G46 7TP.	Acetic acid, acetone, ammonium formate, CDTA, chloroform, Denhardt's, DMSO, EDTA, ethanol, formaldehyde, glycine, HEPES, H_2O_2, KCl, $MgCl_2$, methanol, NaCl, NaH_2PO_4, Na_2HPO_4, NaN_3, NaOH, PB, SDS, sodium acetate, sodium citrate, sodium pyrophosphate, sucrose, Tris acetate, xylene cyanol.
Biorad Laboratories Ltd, Caxton Way, Watford Business Park, Watford, Hertfordshire, WD1 8RP.	APS, Bromophenol blue, 4-chloro-1-naphthol, ethidium bromide, TEMED.
Boehringer-Mannheim, Boehringer-Mannheim House, Bell Lane, Lewes, East Sussex, BN7 1LG.	dATP, dGTP, dTTP, Klenow fragment.
Difco Ltd, PO Box 14B, Central Ave, West Molesey, Surrey.	Trypsin.
Fisons, Gallenkamp, Braeview Place, Nerston, East Kilbride, Glasgow, G74 3XJ.	β-merceptoethanol, glycerol.
Gibco-BRL Ltd, PO Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF.	EcoR1, FCS, formamide, LMP agarose, MEM, NCS, penicillin/streptomycin, REB No. 3, RPMI, urea, WEM.

Koch Chemicals Ltd, 2, Marshgate Drive, Hertford, Hertfordshire.	K_2HPO_4, KH_2PO_4.
Kodak Ltd, Box 33, Swallowdale Lane, Hemel Hempstead, Hertfordshire, HP2 7EU.	Kodak XAR-5 film.
Miles Scientific Division, Miles Laboratories Ltd, Stoke Court, Stoke Poges, Slough, SL2 4LY.	Gelbond film.
Molecular Probes, Inc. PO Box 22010, 4849 Pitchford Ave, Eugene, Oregon 97402.	7-BR, 7-ER, 7-PR, resorufin.
Northumbria Biologicals Ltd, South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL.	DMEM.
Oxoid, Wade Road, Basingstoke, Hampshire.	PBS.
Pharmacia, Pharmacia House, Midsummer Boulevard, Milton Keynes, MK9 3HP.	Dextran sulphate, hexadeoxyribonucleotides.
Scottish Antibody Production Unit, Glasgow and West Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, ML8 5ES.	HRP-anti-rabbit IgG.
Sigma Chemical Company Ltd, Fancy Road, Poole, dexamethasone, Dorset, BH17 7NH.	Acrylamide, BA, BSA, Coomassie blue, cyclophosphamide, diethylpyrocarbonate, L-glutamine, LiCl, 3-MC, 4-morpholine-sulphonic acid, MTT, NADPH, nigrosin, N,N'-methylene-bis-acrylamide, phenol red, proteinase K, protein molecular weight markers, Tris HCl, Tween 20, Type II agarose.
Whatman, Mackay and Lynn Ltd, 2, West Bryson Road, Edinburgh, EH11 1EH.	3MM paper.

Appendix 2.

Publication arising from this project.

Eur. J. Biochem. *174*, 31 – 36 (1988)

Potentiation and suppression of mouse liver cytochrome *P*-450 isozymes during the acute-phase response induced by bacterial endotoxin

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(Received October 27, 1987/January 12, 1988) – EJB 87 1193

Infection and inflammation are known to affect the metabolism and disposition of drugs and carcinogens. We report a detailed study of the effects of bacterial endotoxin on the constitutive and inducible expression and activities of cytochrome *P*-450 isozymes from families *P*-450I, *P*-450IIB, *P*-450IIC and *P*-450III. In general high doses of high endotoxin caused very marked suppression of *P*-450 isozymes and associated activities. However, this effect was differential, the expression of certain isozymes being only slightly reduced whereas others were suppressed to almost undetectable levels. Low doses of endotoxin also gave differential effects on cytochrome *P*-450 expression. Of particular interest was the very marked potentiation of the inductive effect of both 3-methylcholanthrene and phenobarbital. In the case of 3-methylcholanthrene the 10-fold induction of activity was increased to 24-fold by concomitant endotoxin administration. In this regard it was interesting that 3-methylcholanthrene was an effective inducer of a wide variety of acute-phase proteins including metallothionein, serum amyloid A, fibrinogen and hemopexin.

These data show that endotoxin, and therefore bacterial infection and inflammation, can have profound and differential effects on components of the cytochrome-*P*-450 monooxygenase system which could result in significant changes in susceptibility to the effects of drugs, chemical toxins and carcinogens.

Two major systems that are central to protection from ıvironmental insults are the cytochrome-*P*-450-dependent onooxygenase system and the acute-phase response associ-ed with tissue injury and infection. Both of these effects are ɔdiated by genes expressed in the liver.

The mammalian cytochrome-*P*-450-dependent mono-genase system is predominately located in the hepatic endo-ısmic reticulum and consists of a flavoprotein, NADPH – tochrome-*P*-450 reductase, and a family of hemoprotein ›zymes collectively termed cytochrome *P*-450. These talyze the oxidative metabolism of endogenous substrates well as of a large number of structurally diverse xenobiotics –3]. This system is therefore important in defending the st against toxic foreign chemicals by helping to convert :m to forms which can be more readily excreted. Unfortunately, however, cytochrome *P*-450 can also convert relatively innocuous foreign compounds to toxic or carcinogeneic forms [3]. The importance of the cytochrome *P*-450s in xenobiotic metabolism is further underlined by the fact that many forms are induced following exposure of the host to foreign compounds [4].

Endotoxins are lipopolysaccharide components of the outer portion of the cell wall of gram-negative bacteria [5]. The systemic effects of endotoxins on homeostasis by invoking the host's acute-phase response to bacterial infection are well documented and include fever, plasma hypoglycaemia and immunomodulation [6–9]. Hepatocytes respond to endotoxin in a number of ways including a decrease in bile acid secretion, depletion of glycogen reserves and inhibition of both gluconeogenesis and oxygen consumption [10]; stimulation of metallothionein synthesis [11]; and the synthesis of large amounts of acute-phase reactants, notably serum amyloids A and P [12] and C-reactive protein [13]. The increase in synthesis of the above proteins during the acute-phase response is accompanied by a decrease in hepatic synthesis of other exported proteins including albumin [14–16]. Conflicting results have been reported regarding the behaviour of non-exported proteins [17]. Previous reports have indicated that intraperitoneal doses of endotoxin cause a decrease in hepatic microsomal cytochrome-*P*-450-catalysed monooxygenase reactions [18–21]. The cytochrome-*P*-450 monooxygenase system is complex and contains a wide variety of isozymes with different substrate specificities and differing mechanisms of regulation, and the above studies on the effects of endotoxin and interferons on *P*-450 activities have been

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Abbreviations. PB, phenobarbital; MC, 3-methylcholanthrene.

Enzymes. NADPH–cytochrome-*P*-450 reductase (EC 1.6.2.4); ›chrome-*P*-450-dependent monooxygenase (EC 1.14.14.1).

Note. According to the recently reported cytochrome *P*-450 no-ıclature [49] PB_1 is a member of family *P*-450IIC, PB_{2c} family 50III, PB_{3a} family *P*-450IIB, MC_{1a} and MC_{1b} of family *P*-450I. ording to the nomenclature of Ryan et al. [50] PB_{3a} = form b, $_{1a}$ = form d and MC_{1b} = form c. PB_1 is equivalent to PB_1 ·ribed by Waxman and Walsh [51]. PB_{2c} appears to be part of the ;nenolone-16α-carbonitrile-inducible *P*-450 family as reported by :utz et al. [52] and Wrighton et al. [53].

limited in that specific isozymes were not studied. In the present study we demonstrate that endotoxin can have profound and differential effects on mouse hepatic cytochrome *P*-450 isozymes, both in uninduced animals and in animals treated with the *P*-450-inducing agents phenobarbital or 3-methylcholanthrene. The data indicate that infection and inflammation could significantly alter the toxic or carcinogenic effects of environmental chemicals.

EXPERIMENTAL PROCEDURE

Chemicals

Benzphetamine was a generous gift from Prof. F. Oesch (Mainz, FRG). All other chemicals were purchased from commercial sources and were of the highest grade available.

Treatment of animals and preparation of microsomes

Male CBA mice were used throughout and treated with phenobarbital or 3-methylcholanthrene as described previously [22]. Endotoxin was injected intraperitoneally immediately after administration of phenobarbital or 3-methylcholanthrene on the three consecutive days prior to sacrifice (2 μg or 7.5 μg endotoxin/animal, equivalent to 80 μg/kg and 300 μg/kg) or 24 h prior to sacrifice (25 μg/animal, equivalent to 1000 μg/kg). Microsomes were prepared as described previously [23].

Purification of cytochromes and preparation of antibodies

Cytochromes *P*-450 PB_{1a}, PB_{2c}, PB_{3a}, MC_{1a} and MC_{1b} were isolated from either phenobarbital-treated or 3-methylcholanthrene-treated Sprague Dawley rats as described previously [22–25]. All these proteins were of high purity and had specific *P*-450 contents (nmol/mg protein) of: PB_{1a}, 14.7; PB_{2c}, 15.6; PB_{3a}, 15.2; MC_{1a}, 16.4 and MC_{1b}, 20.8 respectively. Antisera were prepared as described previously [26] and used to identify proteins within homologous gene families in the mouse. Induction experiments confirmed the specificity of these antibodies in this regard (not shown).

Western blotting

SDS/polyacrylamide gel electrophoresis and transfer to nitrocellulose were carried out as described previously [27]. Antibody-reactive proteins were visualised by peroxidase staining [27], or by incubation with ^{125}I-labelled *Staphylococcus aureus* protein A (0.1 μCi/ml for 45 min at room temperature) followed by autoradiography. Band intensities were estimated by densitometric scanning of either photopositives (peroxidase blots) or autoradiographs (^{125}I blots) using a Joyce-Loebl Chromoscan-3 densitometer. Three concentrations of each standard were used to assess the linearity of the relationship between band intensity and protein concentration. This analysis, though not strictly quantitative, was found to give a useful subjective measure of the amount of specific protein present.

Enzyme assays and spectral measurements

Cytochrome *P*-450 concentrations were determined by the method of Omura and Sato [28] using an absorption coefficient of 91 mM^{-1} cm^{-1} and cytochrome b_5 was measured by the method of Omura and Takesue [29]. Cytochrome-*P*-450 reductase activity was assayed by a method modified after Phillips and Langdon [30] using cytochrome *c* as substrate. Ethoxyresorufin *O*-deethylase activity was assayed as described by Burke and Mayer [31] and benzphetamine *N*-demethylase activity by a method modified after Hewick and Fouts [32]. Ethoxycoumarin metabolism was measured using the method of Ullrich and Weber [33]. Protein concentrations were determined by the method of Lowry et al. [34].

Northern blot analysis

Total liver RNA was extracted by the guanidinium hydrochloride method [35]. RNA was subsequently fractionated on denaturing formaldehyde gels and analysed as described previously [36]. The cDNA probes were labelled by either the random-priming or nick-translation method [37–39]. Hybridisation and washing were carried out as described previously [36]. The cDNA probes pR17 and pTF1 were a generous gift of Dr M. Adesnik. These probes are for the *P*-450IIB and *P*-450IIC families respectively. pR17 and pTF1 are partial sequences for proteins PB_{3a} and a protein described by Hanui et al. [39] as form f, respectively. The metallothionein probe m_1pEH-4 was a generous gift from R. D. Palmiter.

RESULTS

Spectrophotometric analysis of hepatic microsomal cytochromes and P-*450 reductase*

Treatment of male CBA mice with bacterial endotoxin had differential effects on hepatic cytochrome *P*-450 concentration: a low (2 μg/day) dose of endotoxin did not affect the level of hepatic cytochrome *P*-450 whereas a single dose of 25 μg caused a 39% decrease. This is in agreement with the work of Renton et al. [19] who report approximately 45% loss of hepatic *P*-450 haemoprotein in mice given a dose of 400–4000 mg/kg *Escherichia coli* endotoxin. However, in animals treated concomitantly with endotoxin and phenobarbital, *P*-450 content was only reduced at the highest dose used by 42% and 39% for phenobarbital and 3-methylcholanthrene treatment respectively. Cytochrome b_5 concentration changes followed a similar pattern to the effects observed on cytochrome *P*-450 content (Table 1), with some suppression occurring at the high endotoxin dose.

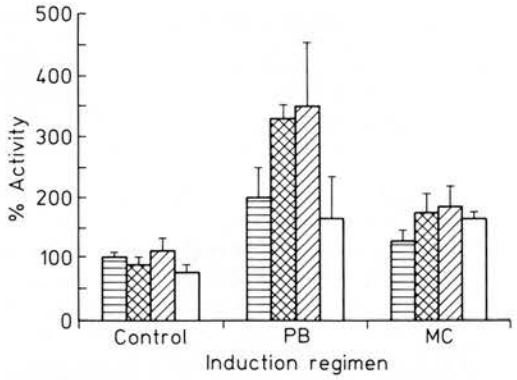
In uninduced animals endotoxin has marginal effects on cytochrome-*P*-450 reductase content (Fig. 1) but when phenobarbital was given with endotoxin (2 μg or 7.5 μg) concomitantly the hepatic cytochrome-*P*-450 reductase activity was greatly increased (1.5–2-fold).

Analysis of cytochrome P-*450-dependent monooxygenase activities*

The effect of endotoxin on cytochrome-*P*-450-dependent monooxygenase activities is shown in Fig. 2a–c. In control mice increasing endotoxin doses generally caused a decrease in the metabolism of the three monooxygenase substrates tested, the only exception being an elevation in 7-ethoxycoumarin metabolism (1.6-fold) at a dose of 2 μg (Fig. 2). At a dose of 25 μg endotoxin, activities were suppressed by 56%, 68% and 76% of benzphetamine, 7-ethoxycoumarin and 7-ethoxyresorufin respectively. In animals treated with phenobarbital a very different pattern of effects on monooxygenase activity was observed, the metabolism of all the

Table 1. *Effect of endotoxin on cytochrome*-P-*450 dependent monooxygenase components*
PB, phenobarbital; MC, 3-methylcholanthrene. Results are means ± SD for 6–9 mice (*P*-450) or 3 mice (b_5)

Endotoxin dose	Cytochrome *P*-450	Cytochrome b_5
	nmol/mg protein (%)	
Control	0.59 ± 0.129 (100)	0.18 ± 0.041 (100)
2.0 μg/day	0.61 ± 0.086 (103)	0.17 ± 0.019 (94)
7.5 μg/day	0.42 ± 0.148 (71)	0.16 ± 0.029 (89)
25 μg (×1)	0.36 ± 0.058 (61)	0.10 ± 0.016 (56)
PB (no endotoxin)	1.14 ± 0.357 (100)	0.28 ± 0.026 (100)
PB + 2.0 μg/day	1.16 ± 0.335 (102)	0.32 ± 0.063 (114)
PB + 7.5 μg/day	1.36 ± 0.221 (119)	0.31 ± 0.050 (111)
PB + 25 μg (×1)	0.66 ± 0.112 (58)	0.25 ± 0.054 (89)
MC (no endotoxin)	0.88 ± 0.286 (100)	0.31 ± 0.016 (100)
MC + 2.0 μg/day	1.02 ± 0.454 (116)	0.26 ± 0.037 (84)
MC + 7.5 μg/day	0.59 ± 0.091 (67)	0.20 ± 0.042 (65)
MC + 25 μg (×1)	0.54 ± 0.263 (61)	0.21 ± 0.019 (68)



g. 1. *Effect of endotoxin on hepatic NADPH–cytochrome*-P-*450-pendent reductase activities in male CBA mice.* Activities are expre-d as percentage of the control, untreated sample (mean ± SD for -9 mice). Control, PB and MC represent samples from uninduced ce or mice treated with phenobarbital or 3-methylcholanthrene pectively. The doses of endotoxin used were from left to right 0, g, 7.5 μg or 25 μg administered as described in Materials and thods. The reductase activities were measured as nmol cytochrome educed min^{-1} mg $protein^{-1}$

bstrates being elevated at lower endotoxin doses. In the case 7-ethoxycoumarin *O*-deethylation the 5.1-fold induction of s activity by phenobarbital was potentiated to 8.1-fold by comitant treatment with endotoxin (2 μg/day). Even ater potentiation of monooxygenase activity by low endo-in doses was observed, for the metabolism of 7-ethoxyre-ufin in animals treated with 3-methylcholanthrene. The uction by 3-methylcholanthrene (10-fold) was increased to fold at an endotoxin dose of 7.5 μg. In both these cases, wever, monooxygenase activity was suppressed by con-nitant treatment with a high dose of endotoxin and in-ing agent, compared with the effect of inducing agent ne. In some cases, however, this effect was small.

stern blot analysis of cytochrome P-*450 apoproteins*

In general uninduced animals were more sensitive to loss nonooxygenase activity caused by endotoxin treatment

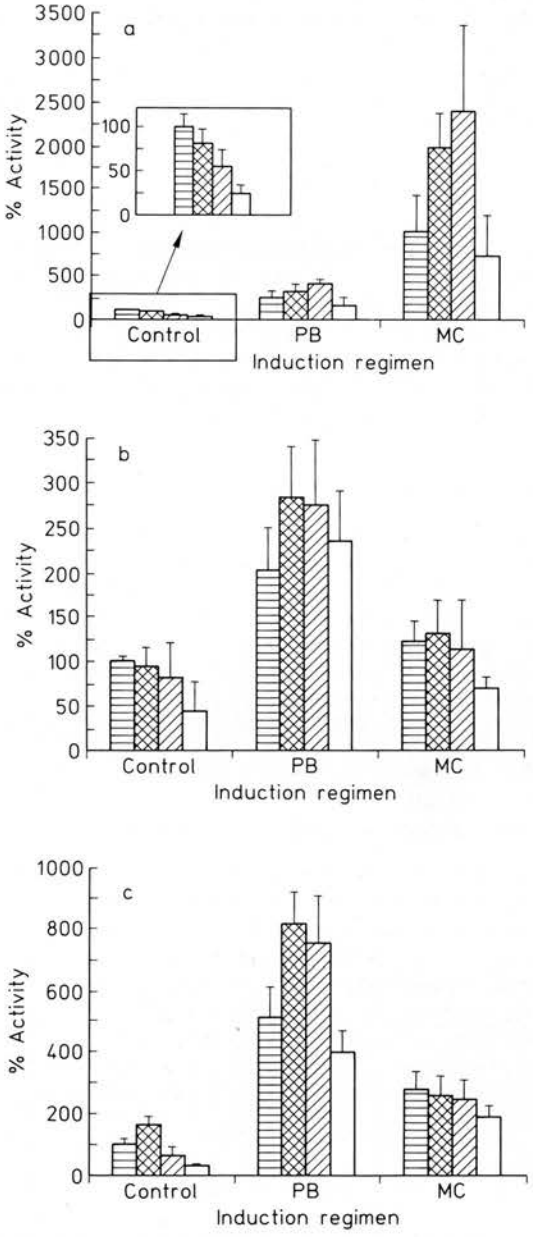


Fig. 2. *Effect of endotoxin on the hepatic cytochrome*-P-*450-dependent monooxygenase activities.* Activities are expressed as mean ± SD for 6–9 mice except for (c) where 3 mice were used. Treatments were as given in Fig. 1 legend. All activitites are expressed as a percentage of the activity of untreated control liver microsomes. (a) 7-Ethoxyresorufin deethylation, (b) benzphetamine demethylation and (c) 7-ethoxycoumarin deethylation. The rates were measured as nmol product min^{-1} mg $protein^{-1}$

than animals treated with phenobarbital or 3-methylcholanthrene. A similar trend was also observed when the relative protein content of some of the cytochrome *P*-450s was determined by Western blot analysis (Figs 3, 4). Low doses of endotoxin caused a slight but reproducible decrease in the content of proteins related to *P*-450 PB_1 in uninduced animals but not in those treated with phenobarbital or 3-methylcholanthrene (Fig. 3a). These data essentially agree with the decrease in constitutive 7-ethoxyresorufin metabolism, which is reported to be mediated by PB_1 [24].

Protein levels related to cytochrome *P*-450 PB_{2c} were not affected by low doses of endotoxin in any of the samples tested

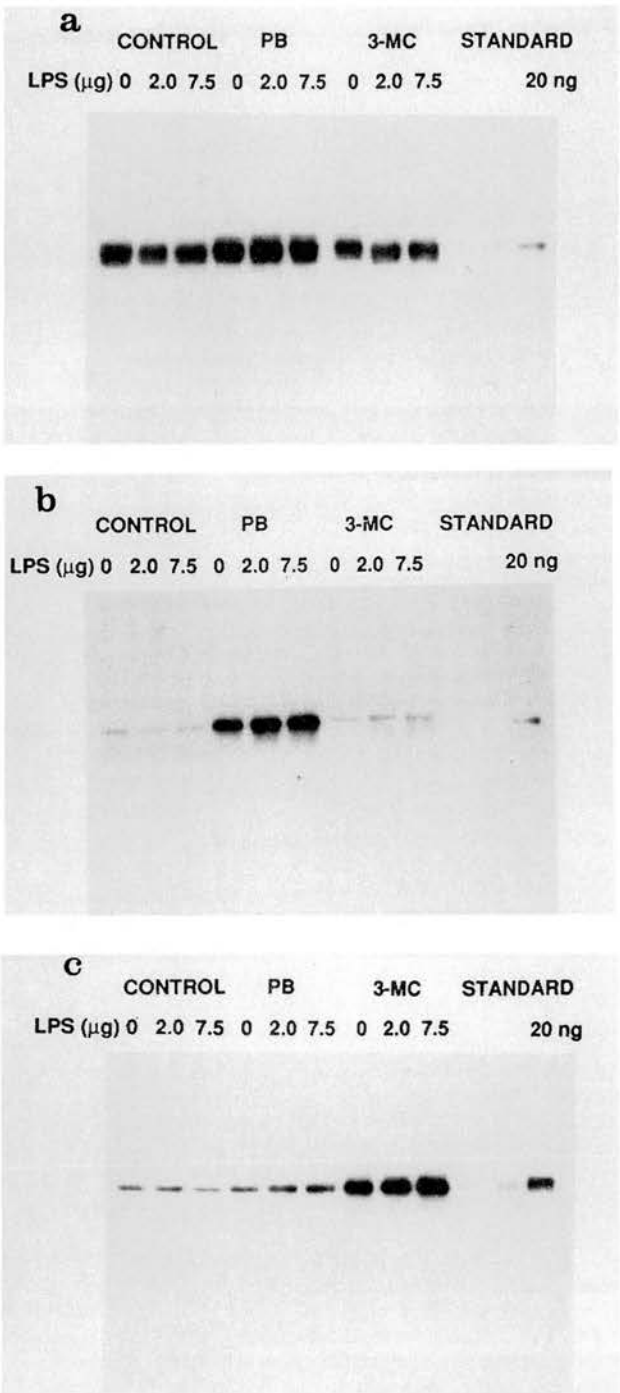


Fig. 3. *Effect of endotoxin on the hepatic expression of isozymes PB_1, PB_{3a} and MC_{1b} in male CBA mice.* Animals were treated with endotoxin and inducing agents as described in Materials and Methods. Liver microsomes from 3 mice were pooled together and analysed by Western blotting (7.5 μg/track). The Western blots shown were with: (a) anti-PB_{1a}, (b) anti-PB_{3a} and (c) anti-MC_{1b}

(data not shown). Constitutive levels of PB_{3a} were extremely low and changes induced by endotoxin were very slight. In samples from mice treated with phenobarbital plus 2 μg or 7.5 μg endotoxin the band intensity was reproducibly higher than those given in phenobarbital alone (Fig. 3b). This correlated well with the increased activity towards benzphetamine (known to be metabolised by PB_{3a}) and 7-ethoxycoumarin in

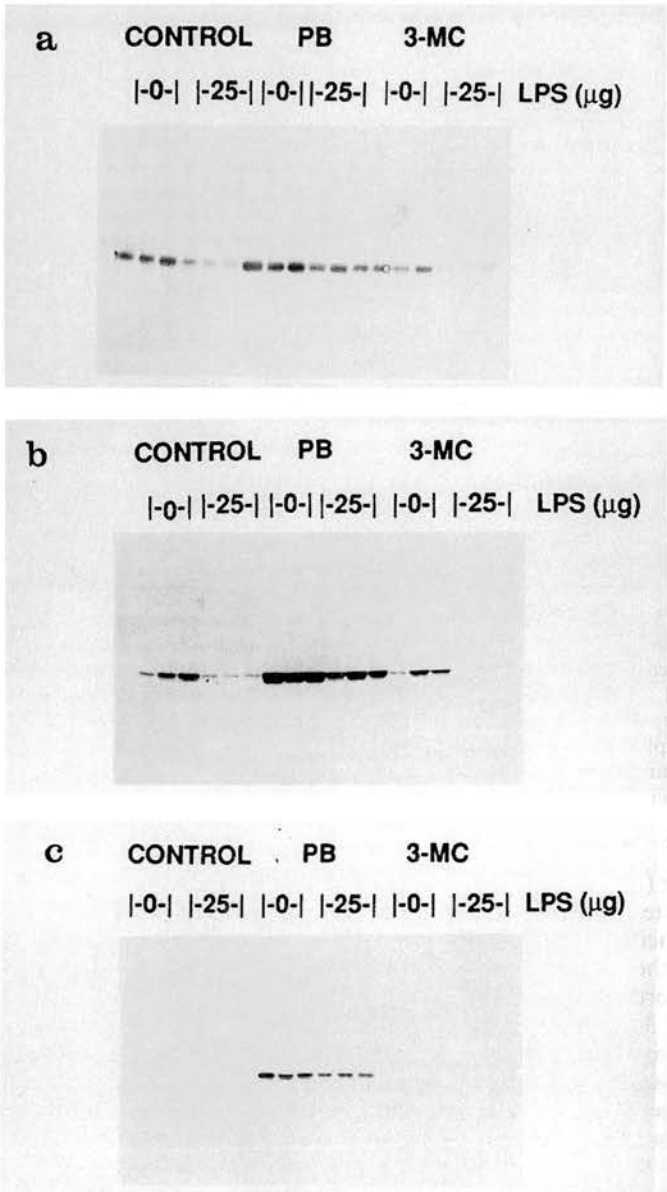
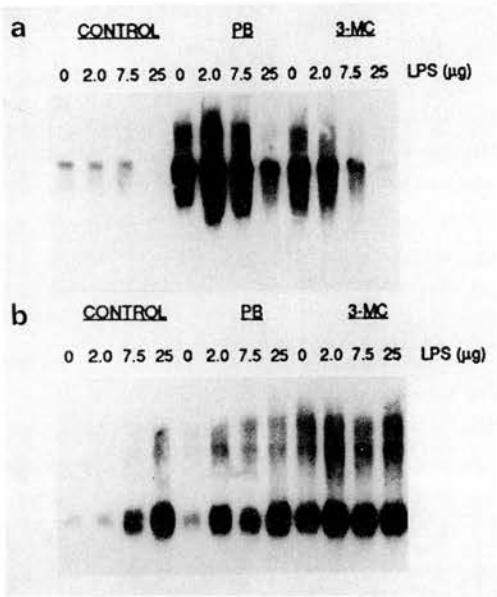


Fig. 4. *Effect of 25 μg endotoxin on the hepatic expression of isozyn PB_1, PB_{2c} and PB_{3a} in male CBA mice.* Mice were treated w inducing agents for three days and endotoxin was administered 2 before sacrifice. Western blotting was carried out on individual mo samples loading 10 μg microsomal protein per track. The blots sho were (a) anti-PB_1, (b) anti-PB_{2c} and (c) anti-PB_{3a}

these samples. Similarly, when low doses of endotoxin w given concomitantly with 3-methylcholanthrene a reprod ible elevation in the level of apoprotein MC_{1b} was observ mirroring the increases in 7-ethoxyresorufin activity measu in these samples.

High doses of endotoxin reduced the expression of all *P*-450 isozymes studied, including proteins related to P PB_{2c} and PB_3, as shown in Fig. 4a–c. PB_1 and PB_{2c} le were very substantially reduced by endotoxin in unindu and 3-methylcholanthrene-treated animals. In agreement w the metabolic data the reduction in PB_1, PB_{2c} and PB_{3a} le was less marked in animals treated with phenobarbital t in uninduced animals. In the blots shown the enzyme P was not detected in control or 3-methylcholanthrene-tre



5. *Quantification of mouse liver mRNA following endotoxin inistration.* Animals were treated as above, and Northern blotting carried out as described in Materials and Methods, loading 10 μg l RNA per track on 1% agarose gels. The cDNA probes used e; pTF-1 (family *P*-450-IIC, i.e. PB_1) (a), metallothionein (b). ples were from control, phenobarbital-treated and 3-methyl-anthrene-treated animals administered endotoxin as described in erials and Methods

(Fig. 3c) although it is present in very low concentration. tern blots, using antisera to the 3-methylcholanthrene-icible isozymes MC_{1a} and MC_{1b}, showed that induction hese isozymes by 3-methylcholanthrene was strongly pressed by the 25 μg dose of endotoxin to values of 41% 34% respectively of the level in samples treated with 3-ylcholanthrene alone. Isozyme MC_{1a} was also sup-sed by endotoxin in uninduced mice (to 23% of control) constitutive MC_{1b} expression was too low to quantify not shown).

hern blot analysis of cytochrome P-*450 cute-phase mRNAs*

n an effort to establish whether the changes observed also reflected in mRNA levels Northern blot analysis was ed out with the available cDNA probes. The experiment ed that the levels of PB_1 mRNA were significantly ressed at high doses of endotoxin in all three test groups 5a). The blot shown was exposed for a long period in to visualize the control mRNA bands. In phenobarbital-d animals mRNA levels were elevated at an endotoxin of 2 μg. The mRNAs for PB_{3a} and MC_{1b} were only ted in the phenobarbital-induced or 3-methylchol-ene-induced animals respectively and did not appear to fected by endotoxin treatment (data not shown).

letallothionein was used as a marker for the acute-phase nse and, in agreement with previous observations, the A for this protein was significantly elevated by endo-treatment (Fig. 5b). It was interesting that metallo-ein mRNA was significantly induced by 3-methyl-nthrene alone. This was also observed for other acute-markers, e.g. serum amyloid A, fibrinogen and hemo-(data not shown).

DISCUSSION

The acute-phase response represents part of a primary defence mechanism against bacterial and parasitic infection and wounding and its effects on the expression of cytochrome *P*-450 isozymes have a variety of implications. At the highest doses of endotoxin used the expression of all the *P*-450 isozymes studied was reduced. For cytochrome PB_1 the mRNA level was also strongly suppressed indicating that a reduced rate of transcription was at least in part responsible. Additional effects such as changes in mRNA stability or elevated rates of protein degradation may also play a role here [19].

The suppression of cytochrome *P*-450 may significantly alter susceptibility to drugs and toxic or carcinogenic foreign compounds, particularly as the effect was isozyme-specific. The suppression of cytochrome *P*-450 and glutathione transferase expression by lymphokines, such as interferons [40–43], interleukin 1 [44] and tumour necrosis factor, has been reported [45]. The extent of cytochrome *P*-450 suppression by endotoxin is more marked than that observed using individual lymphokines [20] (unpublished observations). It is likely, however, that the lymphokines that are induced by endotoxin do play some role in the changes observed.

It was intriguing that, in contrast to the suppression of cytochrome *P*-450 measured at high endotoxin doses, low doses potentiated the expression of certain *P*-450s and related activities. The potentiation of phenobarbital induction could possibly be explained by the increased expression of cytochrome-*P*-450 reductase. However, the expression of the phenobarbital-inducible protein PB_{3a} was also increased. Elevated *P*-450 reductase activity would not explain the profound increase in 3-methylcholanthrene 7-ethoxyresorufin activity on concomitant treatment of mice with 3-methylcholanthrene and endotoxin. The level of MC_{1b} protein was increased in these samples indicating that this may be at least partly responsible for the changes observed.

It has recently been reported that when rats are dosed with dexamethasone in conjunction with 3-methylcholanthrene the induction of MC_{1b} mRNA is potentiated [46]. Endotoxin is known to affect glucocorticoid levels suggesting that a similar mechanism may be involved here. It is also interesting that there is a growing body of evidence indicating structural homology between the receptor which mediates induction by 3-methylcholanthrene and the glucocorticoid receptor [47]. Our studies further substantiate this possibility in that 3-methylcholanthrene induced several acute-phase proteins, some of which are also known to be regulated by glucocorticoids [48]. The exact mechanism and implications of the regulation of these genes by foreign compounds remains to be clarified.

The potentiation of monooxygenase activity by endotoxin implies that infection or inflammation could have significant toxicological consequences, both in the potentiation of drug side-effects and in chemical toxicity. Such effects may also be a factor in chemical carcinogenesis in tissues such as the colon or lung where chronic inflammation occurs together with exposure to carcinogens.

R. R. M. would like to thank ICI Corporate Bioscience Division for financial support and Prof. H. J. Evans for laboratory support and encouragment. We would like to thank Ms A. Ward for her excellent secretarial assistance.

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